1959

Atypical ascorbic acid oxidase of Myrothecium verrucaria

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ATYPICAL ASCORBIC ACID OXIDASE OF *MYROTHECIUM VERRUCARIA*

by

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A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Plant Physiology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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Signature was redacted for privacy.

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Iowa State University
Of Science and Technology
Ames, Iowa

1959

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INTRODUCTION

Oxidases are metal-enzymes which catalyze the direct transfer of electrons from some reduced substrate to molecular O_2 to form water. Their catalytic activity is dependent on the presence of a Cu or Fe-heme prosthetic group attached to the enzyme protein. One such enzyme found widely distributed among higher plants is the Cu-protein, ascorbic acid oxidase. This enzyme has been highly purified and its properties well established (5,16,17,68). A great deal of work has been done on the possible function of ascorbic acid oxidase and the role and metabolism of ascorbate in plant tissues (5,54). It is sufficient to say here that although several routes of electron transfer between certain dehydrogenase systems and ascorbate are known, the significance of these and of ascorbic acid oxidase in synthetic processes or in electron transport to O_2 in intact plant tissues remains obscure.

Recently, two atypical ascorbic acid oxidases were discovered in lower plants. One of these was found by Mandels (51) in spores of the fungus Myrothecium verrucaria and the other by Ward (81) in the slime mold Physarum polycephalum. These enzymes were termed "atypical" in the sense that they differed from the Cu enzyme in response to metal-enzyme inhibitors, reaction products or substrate specificity. As will be presented in this thesis, the

mycelium of M. verrucaria contains an atypical ascorbic acid oxidase with properties distinctly different from those of the Cu enzyme of higher plants, the spore enzyme of Myrothecium and the slime mold enzyme. It was the purpose of this thesis to determine the general properties and cellular location of the mycelial oxidase and to assess what role the enzyme might have as an electron transfer agent in the respiration of the mycelium.

REVIEW OF LITERATURE

Five distinct Cu or Fe oxidase systems in higher plant tissue can oxidize ascorbate. These are ascorbic acid oxidase, polyphenol oxidase, laccase, peroxidase and cytochrome oxidase. Only the former is a true ascorbic acid oxidase since the other systems either require an electron carrier (phenol-quinone or cytochrome c) or depend on hydrogen peroxide as an electron acceptor.

Two different systems are known in which there is a direct reaction between the enzyme, ascorbate and O_2. These are the above mentioned typical Cu oxidase andan atypical, non-Cu enzyme found in spores of the fungus Myrothecium verrucaria (51,52). Another atypical system requiring an intermediate thiol carrier was discovered in the slime mold Physarum polycephalum (81). Neither atypical enzyme has been purified and the nature of their prosthetic groups remains to be determined.

Typical Ascorbic Acid Oxidase

Ascorbic acid oxidase catalyzes the oxidation of ascorbate to dehydroascorbate using one-half mole of O_2 per mole of acid oxidized. Szent-Gyorgyi (76,77) first described the enzyme in 1930 and suggested that ascorbate could function in respiratory electron transport. Since 1930 the oxidase has been isolated and purified from various plant tissues,

among which cucumber (75), pumpkin (18,19) and crook-neck squash (21,65) are particularly rich sources. The enzyme has not been found in mammalian tissues.

Several good reviews of ascorbic acid oxidase purification may be found in the literature (16,17,68). The enzyme is a specific metal-protein having a Cu content proportional to enzymatic activity. Highly purified preparations have been made by workers at Columbia University, where Dunn and Dawson (21) obtained an electrophoretically homogeneous ascorbic acid oxidase from summer crook-neck squash. Their best preparation was a Cu globulin with a molecular weight of approximately 146,000 and a Cu content of 0.26 percent. Conclusive proof that Cu was involved in catalysis was provided by Meiklejohn and Stewart (57), who found that dialysis against cyanide inactivated the enzyme by Cu removal and that the addition of ionic Cu restored 67 percent of the initial activity.

The reported pH optimum values of ascorbic acid oxidases have ranged from 4.6 to 6.5 depending on the type of buffer used, and all have a relatively distinct pH optimum with a range less than 1 pH unit. Purified crook-neck squash enzyme had a pH optimum near 5.6 in phosphate-citrate buffer (65).

A characteristic property of the purified oxidase is enzyme inactivation during reaction, which Powers and Dawson (64) found could be reduced by gelatin, egg albumin and

heme proteins such as catalase or peroxidase. The heme proteins supposedly eliminated a peroxide intermediate or some hydroperoxide complex which inactivates the reactive site during catalysis. Peroxide is definitely not a reaction product (74); and, as Lu Valle and Goddard (49) suggested, perhydroxyl and hydroxyl radicals occur only in ternary complexes and their existence is on the order of 10^{-8} seconds, hence the effect of catalase or peroxidase upon either of these two radicals would be negligible. Catalase and peroxidase may protect the enzyme by stabilizing some intermediate complex which normally causes inactivation.

Frieden and Maggiolo (26,27,29) studied the activation of ascorbic acid oxidase by various proteins, amino acids, nucleotides and metal chelators and found that all activators reacted with cupric Cu. Thyroxine, EDTA, nucleotides, amino acids and proteins activated or protected at all concentrations while cyanide, diethyldithiocarbamate and 8-hydroxyquinoline stimulated at 10^{-7}M and 10^{-6}M but inhibited at 10^{-4}M or higher. These workers also showed that the enzyme was sulfhydryl-dependent and suggested that either free divalent Cu ions or the semiquinone intermediate of ascorbate were formed during the reaction and oxidized adjacent thiol groups inactivating the site. The Cu-complexers appeared to protect reduced thiol groups by reacting with

the active site or chelating freed Cu ions. The latter suggestion does not seem valid considering that the Cu-protein bond is non-dissociable (43).

Another interpretation of reaction inactivation forwarded by Joselow and Dawson (44) was that the protein structure becomes modified during a change in Cu valency and Cu-protein bond angle and becomes an inactive catalytic site. The reversible valency change of the enzyme Cu has recently been demonstrated (28). Since little information exists about the mechanism or intermediates of Cu-enzyme catalyzed reactions, a conclusive explanation of reaction inactivation is impossible at the present time.

The oxidase has been reported to have first order kinetics with respect to ascorbate (56). Frieden and Maggiolo (29) spectrophotometrically determined a K_m of 0.000039M for purified squash oxidase while a considerably higher K_m of 0.0005M was reported for wheat oxidase by Waygood (82). These differing substrate affinities apparently depend on the source of the enzyme.

Copper enzymes are characteristically inhibited by Cu ion chelators. In this respect, ascorbic acid oxidase is strongly inhibited by cyanide, azide, diethyldithiocarbamate, 8-hydroxyquinoline and phenylthiourea. The enzyme is most sensitive to cyanide and diethyldithiocarbamate being completely inhibited at 0.001M (22). Carbon monoxide inhibition

varies considerably depending on the source of the enzyme, but the inhibition is generally weak and light-irreversible. Frieden and Naile (30) found that heavy metal salt and organic mercurial inhibition of the oxidase was reversible with cysteine or reduced glutathione, providing evidence of sulfhydryl group dependence.

The most complete study of ascorbic acid oxidase specificity was made by Johnson and Zilva (42) and Snow and Zilva (73) in England. Johnson and Zilva found that crude cucumber oxidase oxidized at equal rates, L-ascorbate (L-xyloascorbate), D-araboascorbate, L-glucoascorbate and L-galactoascorbate, all of which have the oxygen bridge on the right side of the carbon chain in a Fischer projection. The 6- and 7-carbon analogs, D-xyloascorbate, L-araboascorbate, D-glucoascorbate and D-galactoascorbate were oxidized at approximately one-third the rate of L-ascorbate and at a non-linear rate which decreased with time. The 6-carbon members with the opposite ring configuration to L-ascorbate were oxidized the fastest providing evidence that the rates were determined by the configuration of the assymetric carbon atom in the oxygen ring and the length of the carbon chain. Continuing the previous investigation, Snow and Zilva found that dialyzed cucumber juice oxidized reductone and reductate with rates of O_2 uptake that gradually fell off with time while dihydroxyfumarate was presumably oxidized by polyphenol

oxidase since oxidation occurred only when catechol or phenol was added to the dialyzed extract. Theorell and Swedin (78) disproved the existence of a specific oxidase for dihydroxyfumarate in plant tissues and showed that peroxidase was the enzyme responsible. In contrast to the Cu phenolases, ascorbic acid oxidase does not oxidize mono- and polyhydric phenols (20,50). Dodds (20) extended the number of compounds tested on partially purified cucumber enzyme, finding that imino-D-glucoheptoascorbate, dihydroxyfumarate, dihydroxyacetone, kojic acid, monosaccharic acid and glucuronic acid were not oxidized.

It is clear that the substrate specificity of the Cu enzyme is centered around the dienol grouping. The specificity was not limited to cyclic dienols with an oxygen bridge since reductate and reductone were oxidized. A free carbonyl group adjacent to the dienol group is required since imino-D-glucoheptoascorbate was not oxidized (20). No detailed study of the substrate specificity of highly purified ascorbic acid oxidases has been made, and nothing is known about the nature of substrate attachment to the active Cu-protein site.

Some data has accumulated in the past several years showing that ascorbic acid oxidase can be associated with various fractions of cell homogenates (31,37,61,82). Although the enzyme has usually been considered soluble,

Newcomb (61) found that 90 percent of the oxidase in tobacco pith sedimented with cell wall fragments at 500 x g. Honda (37) presented similar evidence for a peripheral localization of the enzyme in barley roots.

Wide interest has recently been shown in the contribution of oxidases or electron transport pathways other than cytochrome oxidase to cellular respiration in plant tissues. Most of this interest has stemmed from various findings in which the tissue was relatively insensitive to known inhibitors of cytochrome oxidase such as CO, azide or cyanide (32,39,41,79,84), had an apparent low affinity for O_2 (40, 84), or had an apparent lack of extractable cytochrome oxidase or characteristic cytochrome absorption bands. Ascorbic acid oxidase has often been implicated in terminal oxidation in plant tissues although the mass of evidence now available heavily favors cytochrome oxidase in this capacity. There are several lines of evidence which support the conclusion that most, if not all, terminal electron transport proceeds via cytochrome oxidase and not ascorbic acid oxidase. The first of these is that both the intact tissues studied and the extracted cytochrome oxidases from these tissues had a high affinity for O_2 whereas ascorbic acid oxidase had a much lower affinity. The O_2 tension needed for ascorbic acid oxidase to function at maximum velocity is 50-130 times greater than that required for tissue respiration or cyto-

chrome oxidase activity. This, along with the fact that a large amount of cytochrome oxidase can be found in homogenates of these tissues, would suggest that the cytochrome system accounts for all the terminal electron transport under low O_2 tensions. Previously reported low O_2 affinity values for some tissues which have an active cytochrome system were corrected when it was found that respiration rates measured in a liquid phase were limited by the rate of gas diffusion. Higher O_2 affinities were determined by placing the tissues in a moist gas phase thus reducing gas diffusion limitations on respiration. The second line of evidence was that by using the correct assay conditions for cytochrome oxidase, sufficient amounts of the enzyme could be found in homogenates or particulates to adequately support the corresponding respiratory rates in the tissues. The usual cytochrome absorption bands (b, c, a-a_3) can also be seen in slices or bundles of the tissue or in isolated mitochondria. Evidence is rapidly accumulating that the insensitivity of certain tissues to cyanide, azide or CO involves autoxidizable, cyanide-resistant flavoproteins or b-type cytochromes (2,3,6,11,33, 55,84) and possibly a partially cyanide-resistant cytochrome c-a-a_3 system (11), indicating that terminal respiration via cytochrome oxidase and not ascorbic acid xidase occurs in such cases. Unfortunately, inhibitors of ascorbic acid oxidase also affect the cytochrome system providing ambiguous

results as to which is the terminal oxidase in the tissue. Honda (38) has nicely demonstrated this in his studies with barley roots. In summary, there is no evidence so far which would eliminate cytochrome oxidase as the terminal respiratory oxidase in plant tissues. Ascorbic acid oxidase probably does not function in this capacity in tissues which have sufficient extractable cytochrome oxidase and a high affinity for O_2. However, it can not be definitely said that ascorbic acid oxidase never functions in terminal oxidation. Several recent review articles by Bonner (5), Hill and Hartree (34), Mapson (54) and in particular, the one by Smith and Chance (72) elaborate on the evidence discussed above.

Atypical Ascorbic Acid Oxidases

The slime mold Physarum polycephalum contains a soluble, atypical ascorbic acid oxidase which has been described by Ward (81). The chief characteristics of this enzyme were the unusual response to Cu oxidase inhibitors, the requirement for an intermediate electron carrier and the possible formation of peroxide during ascorbate oxidation. The exact nature of the enzyme is not clear, but heavy metal or sulfhydryl groups did not appear to be essential for catalysis since cyanide, azide, CO and several sulfhydryl reagents stimulated the system. A curious feature of the enzyme was that catalytic amounts (0.2 micromoles) of diethyldithiocarbamate

greatly increased the rate of ascorbate oxidation. This effect could be partially induced by tetraethylthiuramdisulfide (oxidized diethyldithiocarbamate) but not by other sulfhydryl compounds. Ward postulated that diethyldithiocarbamate shuttled from the reduced to the oxidized form and acted as an electron carrier between ascorbate and O_2. The fact that parachloromercuribenzoate inactivated diethyldithiocarbamate catalysis gave evidence for a functional thiol group on the carrier. The enzyme was not similar to the sulfhydrylases described by Mandels (53) and Neufeld et al. (59) since only diethyldithiocarbamate was effective as an oxidation promoter and it was not appreciably oxidized in substrate amounts. Cell-free extracts of the slime mold were found to contain a dialysable carrier pigment which restored activity to inactive, dialysed enzyme. Attempts by Ward to isolate and identify this cofactor were unsuccessful. The natural carrier may be diethyldithiocarbamate or a thiol similar in structure.

The O_2 uptake in the diethyldithiocarbamate-catalyzed reaction agreed stoichiometrically with peroxide formation and did not indicate ascorbate peroxidation. Further evidence for peroxide was that added catalase evolved O_2 during the reaction and decreased the total O_2 uptake. However, Ward could not chemically detect peroxide in the completed reaction mixtures. Ward also found that D-araboascorbate

was oxidized at the same rate as L-ascorbate but did not test any other analogs on the system. Since an intermediate electron acceptor is involved, presumably any analog with the same redox potential as ascorbate would be oxidized.

Mandels (51,52) found an atypical ascorbic acid oxidase in spores of Myrothecium verrucaria which differed from the Cu enzyme in response to inhibitors and in substrate specificity. The crude spore oxidase had a wide pH optimum of 4.5 to 7.0 in phosphate buffer and was inactivated during reaction; but unlike the purified Cu enzyme, it could not be protected with gelatin. The stoichiometry of the reaction was one-half mole of O_2 used per mole of ascorbate. The response of the spore oxidase to certain metal oxidase inhibitors suggested that it was not a metal-protein. Cyanide, azide, diethyldithiocarbamate and phenylthiourea stimulated at 0.01M although they completely inhibited the Cu enzyme at this concentration (22,50). The enzyme was inhibited by parachloromercuribenzoate and para-quinone but was stimulated by iodoacetate giving uncertain evidence for functional sulfhydryl groups. Like the Cu enzyme (23, 29,52,79), the reaction rate was limited by low O_2 tensions but was relatively unaffected by substrate concentration.

The spore enzyme had a markedly different substrate specificity than a preparation of ascorbic acid oxidase from squash. Mandels (52) found that the spore enzyme

oxidized L-ascorbate but not D-araboascorbate or D-glucoascorbate, which were oxidized by the Cu enzyme. In spite of the limited number of analogs tested, Mandels' data suggested an absolute specificity for L-ascorbate. The spore enzyme was strongly inhibited by D-araboascorbate. Concentrations as low as 0.00013M gave complete inhibition. Oddly enough, D-glucoascorbate had no similar effect. The results of a fairly detailed study of this inhibition by D-araboascorbate showed that it was not reversed by high concentrations of L-ascorbate and was non-competitive. Further evidence for a non-competitive type of reaction was that the enzyme formed an irreversible complex with D-araboascorbate and O_2. No inhibition occurred unless O_2 was present, leading Mandels to postulate a ternary complex between the enzyme, inhibitor and O_2. D-araboascorbate appeared to react stoichiometrically with the enzyme since a proportionality was observed between the enzyme concentration and the concentration of inhibitor. In addition, the reaction had a positive temperature coefficient and could not be reversed by dialysis or pH treatments. Mandels did not make a kinetic study of the inhibition and much remains to be done with this aspect, particularly on the isolation of the ternary complex and the kinetics of formation.

As previously mentioned, several of the typical ascorbic

acid oxidases are surface-localized (37,61). Mandels provided valid evidence for surface localization of the spore oxidase using indirect methods. His two lines of argument were that pH had the same effect on the intact spore oxidase and the extracted oxidase and that spore exposure to 0.1N HCl for 30 seconds completely inactivated the enzyme without significantly affecting the spore germination, respiration or glucose assimilation. This evidence must be considered indirect since Mandels did not attempt to fragment the spore walls and assay the enzyme distribution between fractions.

No physiological role has been demonstrated for these atypical oxidases although Mandels' finding that the endogenous respiration of spores was not changed when the oxidase was inhibited by acid or D-araboascorbate would tend to eliminate the enzyme in terminal respiration. No data was available on the O_2 affinity of spore respiration or the participation of cytochromes in the spore.

A comparison of the Myrothecium spore oxidase and the slime mold enzyme shows a marked difference between the two. The enzymes have different reaction products, stoichiometry, kinetics and substrate specificities. Inhibitor effects were similar, but diethyldithiocarbamate had no catalytic effect at comparable concentrations in the spore enzyme reaction.

The research in this thesis deals with the general

properties of the atypical ascorbic acid oxidase in the mycelium of M. verrucaria and the substrate specificity of the enzyme compared to that of the spore and Cu oxidases. For the latter study, the enzymes were extracted from spores of M. verrucaria and from fresh cucumber tissue.

MATERIALS AND METHODS

Organism and Culture Technique

Spore culture

The organism used in these studies was the fungus *Myrothecium verrucaria*, strain QM 460, which was received in 1950 from the Philadelphia Quartermaster Laboratories. Cultures obtained from Hilton (35) provided the inoculum for all cultures used during this investigation. The organism was maintained by point inoculation on Whatman No. 1 filter paper supported by a solid agar-salts medium (35) in 125 ml. Erlenmeyer flasks. Before inoculation, the filter paper was sterilized and transferred to hardened medium agar which had been previously sterilized in the flasks. Spores from point inoculations were scraped into 10 ml. of sterile, distilled water and 0.5 ml. of this suspension was used to inoculate solid medium for the production of uniformly aged spores (spore production culture). Spore formation at 30° C was initiated at 2 days and completed at 4-5 days leaving an almost complete mass of black spores on the surface of the solid medium. The possibility of variant strains contaminating the spore production cultures was decreased by using needle-inoculated flasks for spore suspensions. Spore production cultures older than 3 weeks were generally discarded since mycelial growth from these was

usually poor.

Mycelium culture

Mycelial pellets were grown in a liquid medium recommended by Darby and Goddard (14). This had 50 g. glucose, 50 g. Difco malt extract, 2.74 g. $K_2HPO_4.3H_2O$, 2.72 g. KH_2PO_4, 3.00 g. NH_4NO_3 and 2.22 g. $MgSO_4.7H_2O$ per litre of distilled water. The phosphates and the glucose plus nitrate and sulfate were autoclaved separately to avoid magnesium precipitation. Malt extract was cleared twice with filter aid and sterilized by filtration through a bacterial filter. The solutions were then mixed in an automatic pipetter and dispensed in 25 ml. volumes to each of approximately 15, 125 ml. sterile Erlenmeyer flasks. Spores from a 1-3 week old spore production culture were scraped into 10 ml. of sterile, distilled water and a 0.5 ml. inoculum of spore suspension containing approximately 1.7×10^8 spores per ml. was added to each culture and the flasks shaken for 24-30 hours at 30° C on a rotary shaker.

Analytical Methods

Reagents and substrates

The sources of the special reagents and ascorbate analogs used are listed below:

Reagent	Source
Antimycin a	Wisconsin Alumni Research Foundation
2-Hydroxy-3-(2-methyloctyl)-1, 4-napthoquinone (SN 5949)	Abbott Laboratories
Atabrin (Quinacrine.HCl)	Delta Chemical Co.
Isoriboflavin	Dr. T. P. Singer[a]
2,3-Dimercaptopropanol (BAL)	Hynson, Westcott & Dunning, Inc.
Glutathione (GSH)	Schwarz Laboratories
Carbon monoxide (CO)	Matheson Co., Inc.
Flavin adenine dinucleotide (FAD)	Sigma Chemical Co.
Flavin mononucleotide (FMN)	General Biochemicals Inc.
Crystalline liver catalase	Worthington Biochemicals
Cytochrome c	Sigma Chemical Co.
Bovine albumin	The Armour Laboratories
Substrate	
L-Ascorbate	Merck & Co., Inc.
Dehydroascorbate	Nutritional Biochemicals Corp.
D-Glucoascorbate	Nutritional Biochemicals Corp.
D-Araboascorbate (Na)	Hoffman La Roche Inc.
Dihydroxyfumarate	Delta Chemical Co.
D-Xyloascorbate	Prof. F. Smith[b]
L-Araboascorbate	Prof. F. Smith
D-Galactoascorbate	Prof. F. Smith
Imino-D-galactoascorbate	Prof. F. Smith
L-Glucoascorbate	Prof. F. Smith and Prof. J. K. N. Jones[c]
Imino-D-glucoascorbate	Dr. G. Reynolds[d]
Imino-L-guloascorbate	Dr. G. Reynolds

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[a]The Ford Institute for Medical Research.

[b]University of Minnesota, St. Paul, Minnesota.

[c]Queen's University, Kingston, Ontario, Canada.

[d]Eastman Kodak Co., Rochester, New York.

Substrate	Source
6-Deoxy-L-ascorbate	Prof. T. Reichstein[e]
L-Rhamnoascorbate	Prof. T. Reichstein
L-Galactoascorbate	Prof. T. Reichstein
L-Guloascorbate	Prof. T. Reichstein
L-Erythroascorbate	Prof. T. Reichstein
Hydroxytetronate	Prof. T. Reichstein
Reductate	Dr. Gerhard Hesse[f]
Methyl reductate	Dr. Gerhard Hesse
Reductone	Dr. J. R. Holker[g]

[e]University of Basel, Switzerland.

[f]University of Freiburg, Germany.

[g]Tootal Broadhurst Lee Co., Manchester, England.

Reagents were weighed on an analytical balance and dissolved or suspended in 0.05M potassium phosphate-0.025M citrate buffer and adjusted to the required pH with 10 percent NaOH or 0.1N HCl using a Beckmann meter. Antimycin a and SN 5949 were dissolved in 95 percent ethanol to give stock solutions of 200-400 micrograms per ml. which were kept at 4° C. The maximum amount of these solutions used per flask was 0.1 ml. in a total volume of 2.0 ml. (5 percent) with an equal volume of ethanol added to each control. The catalase was dialysed against distilled water for 24 hours to remove ammonium sulfate then was diluted 100 times with distilled water to give an enzyme solution containing about 0.25 catalase units per ml. The ascorbic acids available in quantity were made up in 0.05M phosphate-0.025M citrate buffer and adjusted to the required pH with 10

percent NaOH. Smaller samples of ascorbate analogs were dissolved in phosphate-citrate buffers of varying strengths to give a pH of 5.0-5.5. Autoxidation of L-ascorbate at pH 4.5 in air was generally negligible and never more than 10 microliters of O_2 per hour. However, glutathione (GSH) was added in sufficient amounts with all substrates to reduce autoxidation. Autoxidation controls with boiled enzyme or without enzyme were run when necessary, for example, in reactions involving high pH, high O_2 tension, stoichiometry and certain ascorbate analogs.

Extraction of the mycelial, spore and Cu enzymes

A standard procedure for enzyme extraction from the mycelium was developed. The procedure involved filtering the mycelial pellets under suction, washing twice with 100 ml. of distilled water, then washing the mycelial mat with 200 ml. of cold, pH 6.3, 0.01M phosphate-0.005M citrate buffer. Following filtration, the wet mat of approximately 12 g. was sectioned into small pieces and ground in a Potter-Elvehjem tube with an equal weight of 100 mesh pyrex glass and 15 ml. of cold, pH 6.3, 0.01M phosphate-0.005M citrate buffer. Three individual grinds were made in an ice bath using about 20 passes of the pestle per grind. After each grind, the homogenate plus washings from the pestle and tube were centrifuged in a pre-chilled 50 ml. glass tube for 10 minutes at 1000 x g. at 0° C. The

supernatant was decanted and stored at 4° C in 25 ml. plastic centrifuge cups and the residue returned to the grinding tube. A final spin of the cell-free extract for 30-40 minutes at 20,000 x g. at 0° C gave an opaque extract. The total final volume of approximately 60 ml. was pipetted from the particulate residue and stored frozen.

Prior to measuring activity, the extract was acidified to pH 4.5 with 1N HCl. This step flocculated an insoluble fraction which could be separated by centrifugation at 1000 x g. Both the soluble and insoluble fractions oxidized ascorbate. The insoluble fraction was resuspended in cold, pH 4.5, 0.01M phosphate-0.005M citrate buffer when tested.

Spore extracts were prepared by washing a suspension of spores from 5 spore production cultures twice with distilled water at the centrifuge. The wet spores of approximately 4 ml. volume were then ground twice in a Potter-Elvehjem tube with cold, pH 6.2, 0.01M phosphate-0.005M citrate buffer and 12 g. of 100 mesh pyrex glass. The debris was spun down for 10 minutes at 500 x g. at 0° C and the supernatant decanted into 25 ml. plastic centrifuge cups. After centrifuging for 30 minutes at 20,000 x g. at 0° C, the opaque extract containing the enzyme was decanted and diluted to 20 ml. with cold buffer and stored frozen.

Fresh cucumbers were bought from the local grocery and

stored in the cold until sliced. Thin cucumber slices were frozen at -14° C overnight and then allowed to slowly thaw and autolyze in a Buchner funnel at room temperature. The crude juice obtained in this way was collected in a pyrex tube immersed in crushed ice and was used immediately in the experiments after the activity of the enzyme was determined and dilutions made when necessary with cold, pH 6.3, 0.01M phosphate-0.005M citrate buffer to give a rate of 200-400 microliters of O_2 uptake per hour.

Intact mycelium preparation

Two types of intact mycelium were used, starved mycelium in buffer and unstarved mycelium disks exposed to a moist air phase. For starved mycelium, 8 cultures were filtered, washed once with distilled water, then resuspended in 100 ml. of cold, pH 6.3, 0.01M phosphate-0.001M Mg buffer and filtered. This washing step was repeated and the mat resuspended in 150 ml. of the buffer. The mycelium was shaken and starved in a 250 ml. Erlenmeyer flask for 28-30 hours at 30° C. After starving, the culture was filtered, washed once with distilled water and redispersed in 60 ml. of cold distilled water plus 0.001M Mg, using a nickel spatula. Mycelium prepared in this way was used to study the effect of pH on the surface-localized enzyme. One ml. of the suspension was used in each Warburg flask containing 0.8 ml. of reaction mixture and 0.2 ml. of 20 percent KOH in

the centre well to absorb CO_2. Endogenous respiration either was measured initially before tipping in ascorbate or by including at least 3 controls without ascorbate for the time of measurement. Dry weight determinations were made on duplicate 5-10 ml. aliquots of suspension filtered on tared, sintered glass crucibles and dried at 100° C for 24 hours.

A method was developed for preparing mycelium for respiration studies in a moist gas phase. Pellets from 12 flasks were filtered and washed twice with 100 ml. of distilled water then resuspended in 100 ml. of cold, pH 6.0, 0.01M phosphate buffer. After filtration, the mat was dispersed in 200 ml. of the buffer. Sixty ml. of this suspension was diluted to 100 ml. with buffer and suction-filtered on 11 cm. Whatman No. 1 paper using a "sandwich" mat of ground filter paper pulp and filter paper underneath. This procedure was found necessary to prevent the mycelium from clumping around the pores of the Buchner funnel. The thin mat of mycelium was kept under suction until nc more water came through and was then peeled off and placed on a dry filter paper. About 20 disks of 14 mm. diameter were cut out with a cork borer and placed in a dry Petri dish. The disks were allowed to dry in air until they assumed a pale white color. This procedure removed surface water from the disks but did not reduce the endogenous respiration rate. After drying, 3-5 disks of about 0.5 mm. thickness were

placed in the bottom of each Warburg flask having 0.2 ml. of 20 percent KOH in the centre well and a small filter paper wick plus 0.3 ml. of water in the side arm to prevent disk dessication during respiration measurements. Dry weights were determined for each set of disks after each run. Respiration rates were compared on the basis of microliters of O_2 uptake per mg. dry weight of mycelium per hour (Q_{O_2}).

Dialysis

The method used for enzyme dialysis against cyanide was essentially that of Meiklejohn and Stewart (57). Soluble and insoluble enzyme fractions were prepared as previously described, the insoluble fraction being washed once with 10 ml. of cold, pH 4.5, 0.01M phosphate-0.005M citrate buffer and then centrifuged and resuspended in the same volume of buffer. Dialyses were done at 8°C in cellophane sacks which were slowly swirled through the dialysing solution. Both fractions were dialyzed for 36 hours against 400 ml. of pH 4.5, 0.01M KCN in 0.01M phosphate-0.005M citrate buffer. A dithizone test for Cu in the dialyzing solution was negative. Following this step, the sacks were washed free of external cyanide and placed in 400 ml. of cold, cyanide-free, pH 4.5 phosphate-citrate buffer. One 400 ml. buffer change was made during a total dialysis time of 48 hours when no cyanide could be detected by the Cu

sulfide speck test described by Fiegl (25). The volume of both fractions was measured and enough solid $CuSO_4$ added to give a 10^{-5}M concentration of Cu ions. After incubating overnight at 8° C, the excess Cu was dialysed out against a total of 2000 ml. of pH 4.5, 0.01M phosphate-0.005M citrate buffer. Metal ions other than Cu and flavin nucleotides were added to the cyanide-free fractions 10 minutes before the activity determination.

Gas mixtures and measurement of O_2 uptake

Gases were mixed at atmospheric pressure by displacing measured volumes of water in 4 litre containers. Carbon monoxide and O_2 were used directly from the tank. The commercial nitrogen was routinely washed with alkaline pyrogallol to remove O_2. Most of the gassing of Warburg flasks was done outside the bath using a flow rate of about 2 litres of gas per 5 minutes through duplicate flasks. Aluminum foil was wrapped around the flasks in the CO-dark studies.

All reaction measurements were made at 30° C in a Warburg respirometer having a shaking rate of 130 oscillations per minute. Depending upon the volume, either the substrate or enzyme was placed in the side arm and tipped into the reaction mixture after a 10 minute equilibration in the bath. Readings were generally taken at 5 minute intervals and the initial rates were calculated from drawn reaction curves in all experiments. As developed in detail in the

next section, the standard reaction system contained enzyme extract, 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate in a total fluid volume of 2.0 ml. In order to use smaller amounts of the rarer ascorbate analogs, the same relative concentrations of the standard reaction components were used in a fluid volume of 0.5 ml. in 5.0 ml. Warburg flasks. Alkali was not included in the flasks except for mycelial respiration studies. Final reaction pH's were measured with a Beckmann meter.

EXPERIMENTAL RESULTS

General Properties of the Mycelial Enzyme and Development of Reaction Conditions

Ascorbic acid oxidase in the mycelium was first found when ascorbate was added to the high-speed supernatant from a particle preparation. The grinding and extraction method used was essentially that developed by Hilton (35) for particles, the only modifications being, first, to omit the washing of the mycelium in a blendor which resulted in a loss of oxidase activity and, second, to use a 0.01M phosphate-0.005M citrate extraction medium. No particular effort was made to obtain a maximum enzyme yield since the grinding was not complete. Generally, it was found that 2 grinds with 12 g. of wet mycelium and a total extract volume of 60 ml. would give initial rates of 150-400 microliters of O_2 per hour with 0.8 ml. of extract per flask. The enzyme was stable to incubation at 30° C in air or O_2 but was completely inactivated by heating to 100° C for a few seconds. Preparations stored frozen at pH 6.0 for several months showed no activity loss.

Grinding the mycelium in buffers of pH 5.0 or lower resulted in some activity loss due to the precipitation of an insoluble fraction carrying from 20-70 percent of the total ascorbic acid oxidase activity. This was shown when acidification of a pH 6.2 supernatant from a 20,000 x g.

spin precipitated the fraction. An example of this is given in Table 1. As previously described in the Methods section, the insoluble fraction was separated by low-speed centrifugation in the cold and was resuspended in pH 4.5, 0.01M phosphate-0.005M citrate buffer. The fractionated supernatant is referred to as the soluble fraction.

Table 1. Effect of washing on the insoluble fraction.

Expt.	Fraction	Treatment[a]	Activity[b] (ul. O_2/hr.)
	Extract	Adjust to pH 4.5	163
	Soluble	-	85
1	Insoluble	Resuspend in buffer	84
	Insoluble	1st wash in buffer	72
	Insoluble	2nd wash in buffer	78
2	Insoluble	Resuspend in buffer	204
		Supernatant from 1st wash in buffer	30

[a]Washing and resuspension in pH 4.5, 0.01M phosphate-0.005M citrate buffer.

[b]Activity based on equal aliquots of each fraction. Rates for the insoluble fraction are expressed on the basis of activity in the original extract. Each flask contained 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate.

The first experiments with the insoluble fraction were to find if the enzyme was merely carried down at pH 4.5 by other proteins or whether the enzyme was actually insoluble. Two experiments are given in Table 1 in which the insoluble fraction was washed with pH 4.5, 0.01M phosphate-0.005M

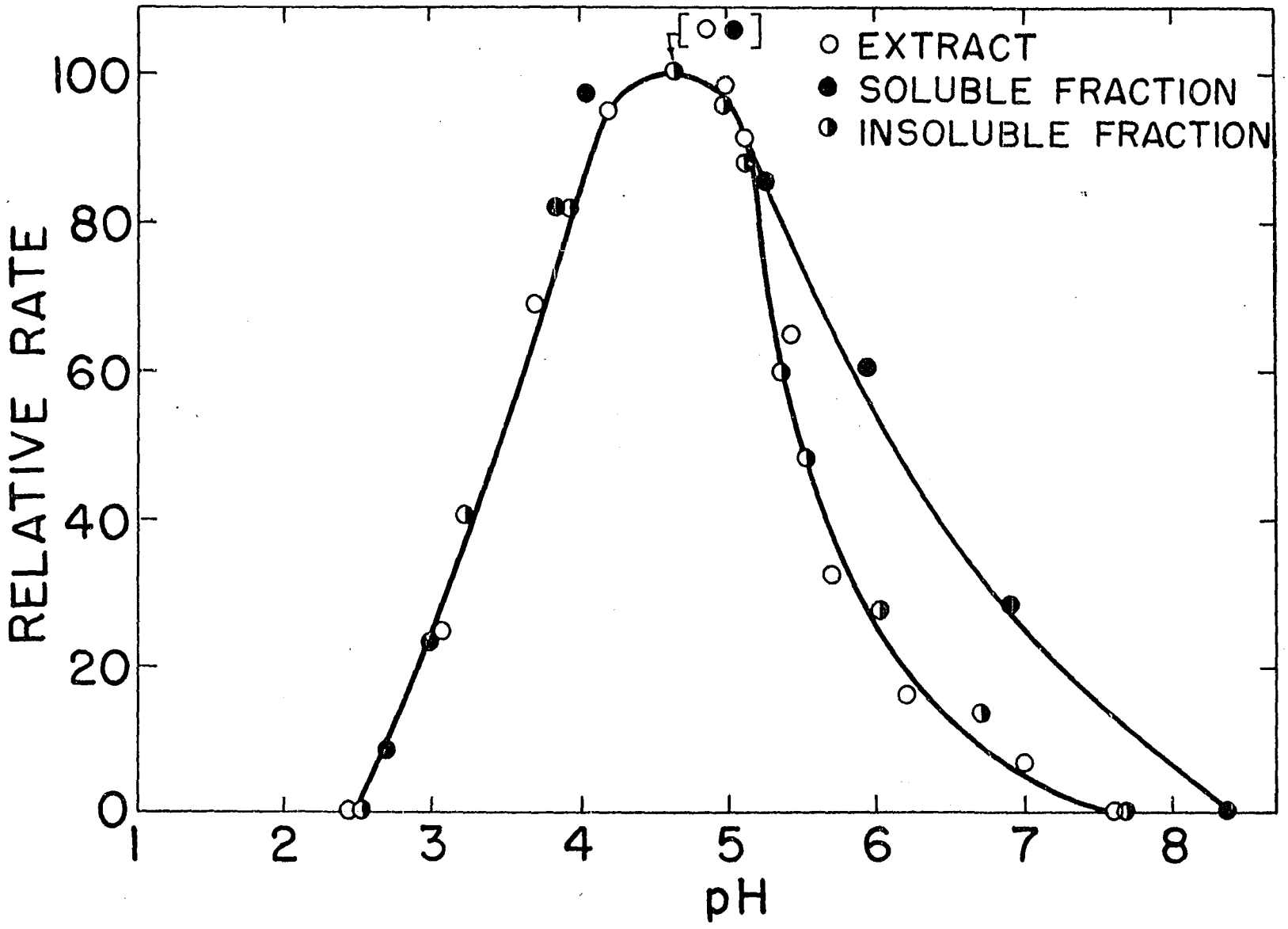
citrate buffer. About 15 percent of the total activity in the insoluble fraction was removed by a single washing but further washing had no effect. This small amount was likely absorbed from the supernatant during precipitation and does not represent resolubilized enzyme since a continued decrease in activity would be expected after successive washes.

Possible participation of oxidases other than ascorbic acid oxidase was considered, and tests were made for polyphenol oxidase and for cytochrome oxidase in the high-speed supernatant. Substrate amounts (3 mg.) of p-cresol and L-tyrosine were not oxidized at pH 6.0, indicating a lack of polyphenol oxidase and tyrosinase. Cytochrome oxidase activity could not be detected using 3 mg. of hydroquinone and 1 mg. of cytochrome c. In addition, 1 mg. of cytochrome c did not stimulate ascorbate oxidation. Qualitative tests showed high catalase activity. These tests and the results presented later with Fe and Cu enzyme inhibitors agreed that ascorbic acid oxidase alone was oxidizing the added ascorbate.

The effect of several phosphate-citrate buffer concentrations on the reaction rate showed that molar strengths higher than 0.04M phosphate-0.021M citrate inhibited the enzyme. Consequently, a buffer strength of 0.034M phosphate-0.017M citrate was chosen to give maximum buffering at pH 4.5. The variation in activity with pH was then studied using this buffer concentration. Figure 1 shows the pH-

Figure 1. Effect of pH on oxidation of ascorbate by the enzyme extract and the soluble and insoluble fractions.

Reaction conditions: 0.0112M ascorbate, 0.034M phosphate-0.017M citrate.

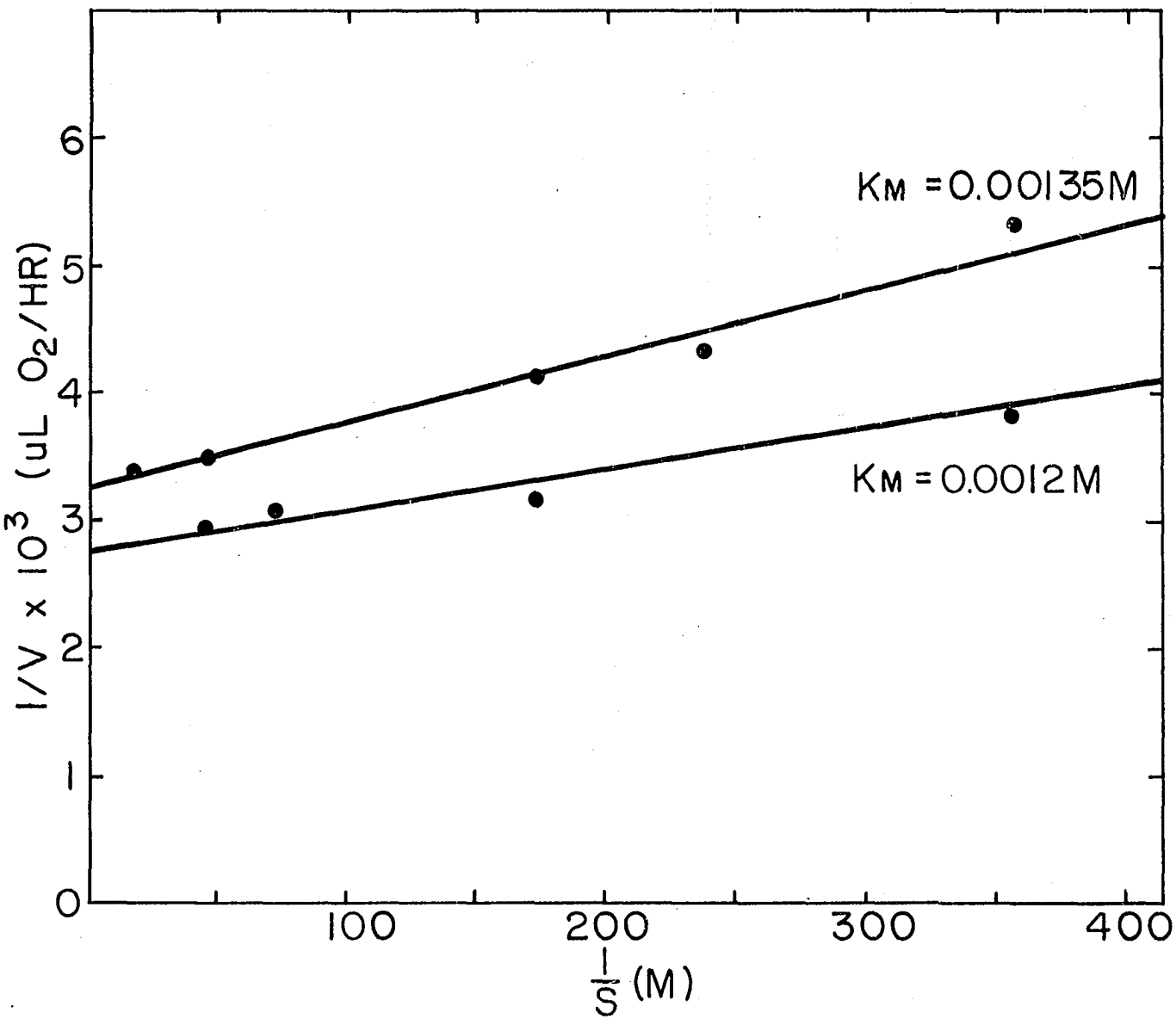

EXTRACT
SOLUBLE FRACTION
INSOLUBLE FRACTION
RELATIVE RATE
100
80
60
40
20
0
1
2
3
4
5
6
7
8
pH

activity curve of the extract and both fractions. The enzyme was added to buffered reaction mixtures and the final pH was measured with a Beckman meter. Phosphate buffer alone produced the same pH response as phosphate-citrate with little variability among experiments. The observed pH optimum of 4.2 to 4.8 was independent of substrate concentration over a tested range of 0.0028M to 0.028M ascorbate. The activity curve of the soluble fraction was consistently skewed to the alkaline side while the extract and the insoluble fraction had identical bell-shaped curves. A pH-stability curve was not run and little data is available on this question except that rapid, irreversible acid inactivation was found at pH 2.5. A further treatment of the pH curves will be given in the discussion section.

After the pH optimum was determined, a study was made of the effect of ascorbate concentration on the reaction rate of the enzyme. The rate was found to increase rapidly to about 0.004M and then sloped off and reached a constant maximum value above approximately 0.015M ascorbate. A Lineweaver-Burk (47) plot of the data from two such experiments with the enzyme extract is shown in Figure 2. In a total of 5 experiments, the calculated apparent K_m was observed to range from 0.00115M to 0.00135M. In a single experiment the soluble and insoluble fractions had the same apparent K_m as the extract. No deviation from Michaelis

Figure 2. Lineweaver-Burk reciprocal plot of reaction rate as a function of ascorbate concentration.

Reaction conditions: pH 4.5, 0.034M phosphate-0.017M citrate.


Km = 0.00135M
Km = 0.0012M
1/V x 10^3 (uL O2/HR)
1/S (M)
0
1
2
3
4
5
6
100
200
300
400

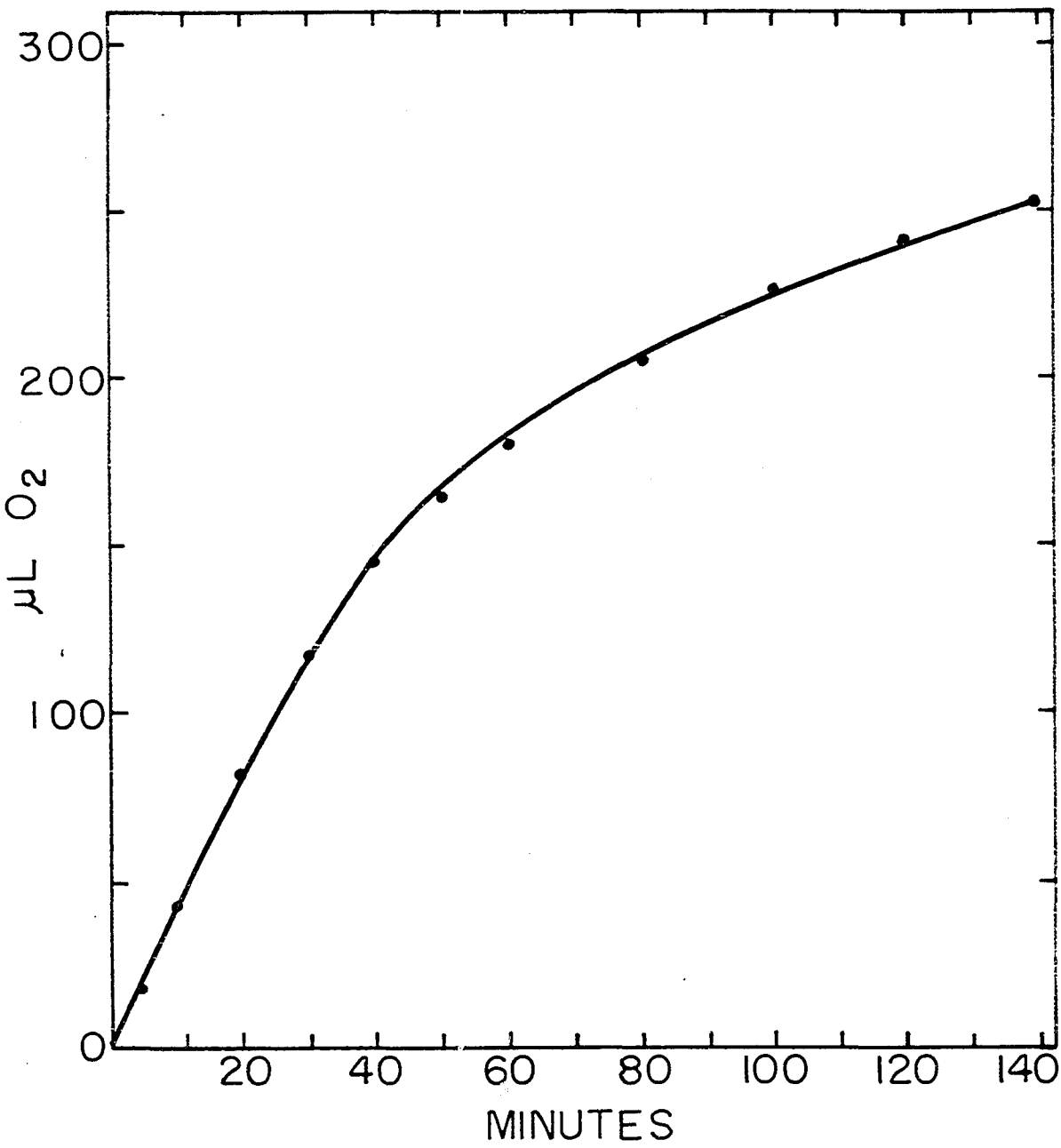
kinetics (58) was observed at the higher substrate concentrations indicating that ascorbate does not act as an inhibitor or an activator. As a result of these tests, the range of ascorbate concentrations chosen for the standard assay was 0.0112M-0.0140M (4-5 mg. per 2.0 ml.).

On the basis of the previous experiments, the standard experimental conditions chosen were: 0.8 ml. enzyme extract, 0.0112M-0.0140M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate, and a shaking rate of 130 oscillations per minute at 30° C in air. The development of these assay conditions allowed a quantitative study of the properties of the enzyme. However, as shown in the next section, the reaction conditions were not optimal since the rate in air was limited by O_2 tension.

Under the standard assay conditions, the reaction curves for the enzyme were approximately linear for only 10-15 minutes then fell off gradually until stoichiometry was reached. This is shown in Figure 3 which illustrates why the rates of O_2 uptake were expressed as initial reaction velocities. The decrease in rate with time was not due to substrate depletion since the same effect was found at high concentrations of ascorbate (0.028M). As presented and analyzed in some detail in the next section, the fall-off in rate was caused by inactivation of the enzyme during reaction (reaction inactivation).

Figure 3. Typical reaction curve for the oxidation of ascorbate by the mycelial enzyme.

Reaction conditions: 0.0140M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate. Theoretical stoichiometry was 320 microliters of O_2.


300
200
100
0
µL O2
20
40
60
80
100
120
140
MINUTES

The rate of O_2 uptake was proportional to the amount of enzyme extract as shown for a typical experiment in Figure 4. This same linear relation also was found for both enzyme fractions. The proportionality of rates to enzyme concentration extended up to initial rates of 300-350 microliters of O_2 per hour. No explanation for the non-proportionality at the higher rates can be given except that it does not appear to involve O_2 diffusion, ascorbate concentration or inhibition by dehydroascorbate.

Similar to the Cu oxidase (74) and the spore enzyme (51), the stoichiometry agreed with an uptake of one-half mole of O_2 per mole of ascorbate oxidized. Table 2 gives the autoxidation-corrected values obtained for 2 experiments with different enzyme preparations. The variability in percentage of theoretical uptake among experiments was negligible and usually within 5 percent of the calculated theoretical value assuming one-half mole of O_2 used per mole of ascorbate. Dehydroascorbate was not oxidized since no further O_2 or CO_2 gas exchange was found after the stoichiometric amount of ascorbate was oxidized. The addition of dialysed crystalline catalase or inhibiting the endogenous catalase activity with azide had no effect on the stoichiometry as it did in Ward's system (81), providing evidence that peroxide was not a final reaction product. However, the fact that catalase was without effect

Figure 4. Proportionality of initial rates of O_2 uptake to amount of enzyme extract.

Reaction conditions: 0.0140M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate.

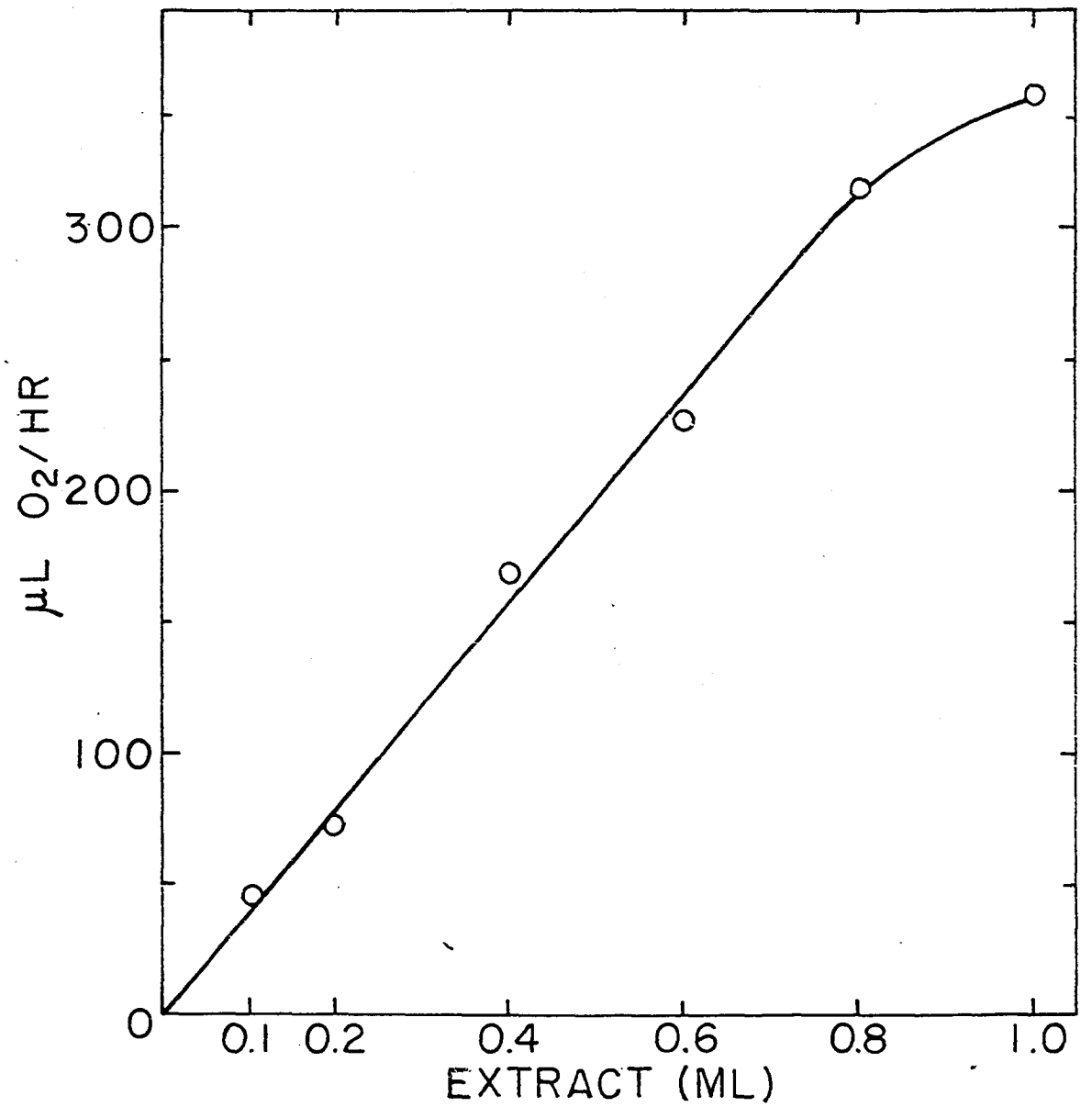

300
200
100
0
μL O2/HR
0.1
0.2
0.4
0.6
0.8
1.0
EXTRACT (ML)

Table 2. Stoichiometry of the mycelial enzyme on the basis of one-half mole of O_2 used per mole of ascorbate.

Experiment[a]	Ascorbate (mg.)	Maximum ul. O_2 uptake		
		Obs.	Calc.	Theor. (%)
1	0.0	negl.	---	---
	0.8	51	51	100
	1.0	68	64	106
	2.0	136	128	106
	3.0	194	192	101
2	0.0	---	---	---
	1.0	63	64	99
	2.0	128	128	100
	3.0	196	192	102
	4.0	248	256	97

[a]All flasks contained pH 4.5, 0.034M phosphate-0.017M citrate. Observed values were corrected for autoxidation.

does not eliminate the possible formation of an intermediate enzyme-peroxide complex.

Kinetics of the Mycelial and Spore Enzymes

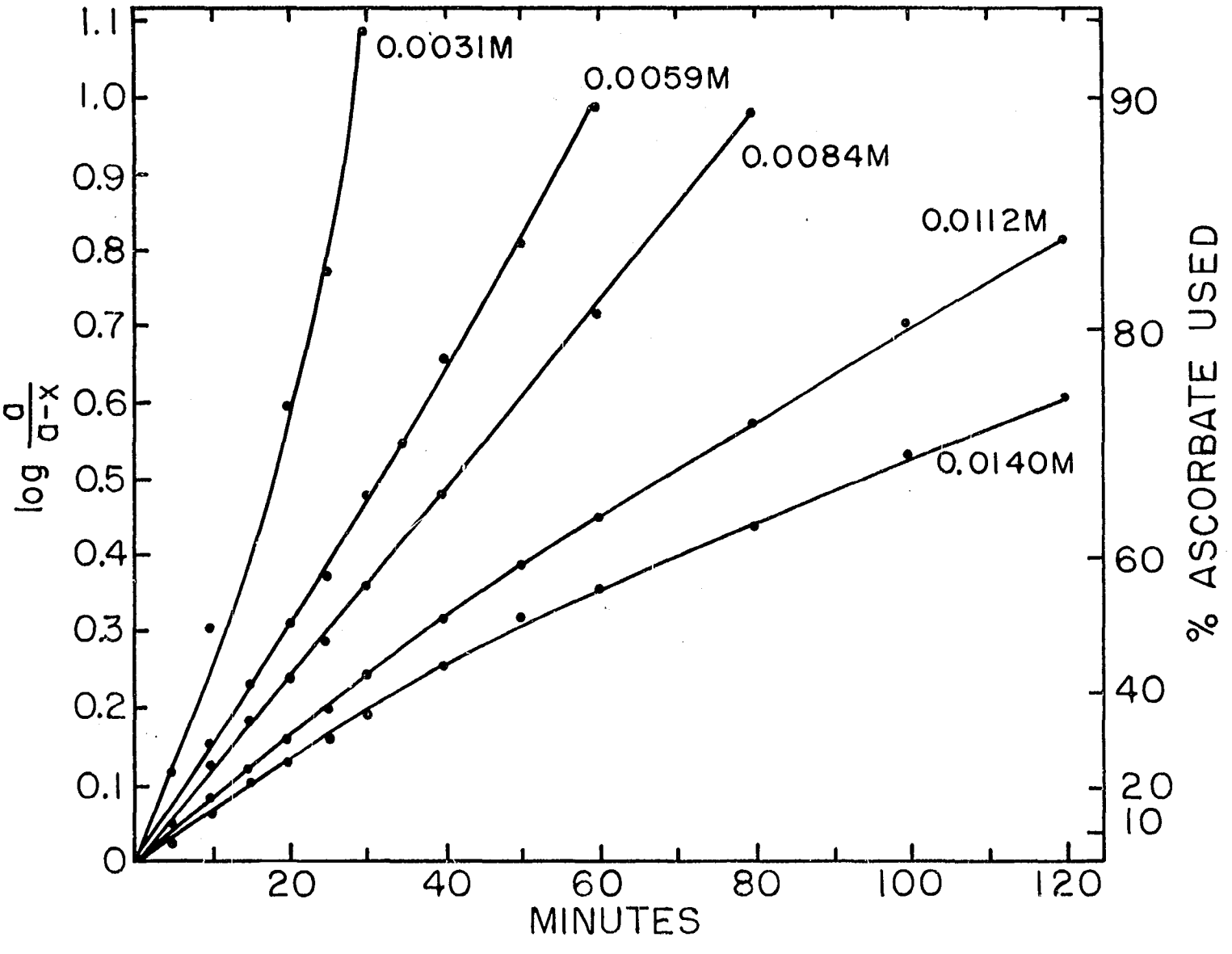
The kinetics of the mycelial and spore enzymes was investigated with the purpose of determining the apparent reaction order with respect to the substrate and to the enzyme. This was done by measuring the reaction rates at various ascorbate concentrations and analyzing the resulting rate curves by several kinetic methods. In addition, the kinetics of the mycelial enzyme in O_2 were studied since the concentration of the third reactant was obviously important in such an analysis.

Mycelial enzyme

The general shape of the rate curves for the mycelial enzyme such as that in Figure 3 indicated that the reaction order was not zero with respect to ascorbate since a plot of x (amount of ascorbate used in terms of O_2 uptake) against time failed to give a straight line. If the reaction were first order with respect to ascorbate, then a straight line (independent of substrate concentration) would be obtained on a first order log plot such as the one in Figure 5 for the mycelial enzyme in air. It can be seen from the figure that a plot of the data according to the conventional first order equation yielded curves of non-linear relations over a substrate range of 0.0031M to 0.0140M. As the concentration of substrate decreased, the curves began to approximate a linear (first order) course while at the higher levels of substrate, the velocity increased with time more rapidly than is required by first order kinetics then deviated in the opposite direction and simulated second order kinetics. It was also found from curves such as in Figure 5 that the first order reaction constant calculated from the linear part of the curves increased with a decrease in substrate concentration. The reactions were followed out to approximately 90 percent substrate utilization since the initial period of reaction does not usually allow the determination of reaction order. Similar types of curves

Figure 5. First order reaction plot of ascorbate oxidation by the mycelial enzyme in air.

Reaction conditions: pH 4.5, 0.034M phosphate-0.017M citrate. Molar concentration of ascorbate is given with each curve. a: initial amount of ascorbate, x: amount of ascorbate used in time t.


0.0031M
0.0059M
0.0084M
0.0112M
0.0140M
log a/a-x
% ASCORBATE USED
MINUTES
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
10
20
40
60
80
90
20
40
60
80
100
120

were found when the enzyme concentration was varied at a constant substrate concentration. These showed an increase in initial slope as the time needed for 90 percent reaction was decreased. It was evident from these results that an analysis of the data by first order kinetics was not valid even at low substrate concentrations since zero order effects were present and second order effects were introduced by enzyme inactivation.

If the rate-determining step is the conversion of the enzyme-substrate complex to reaction products and free enzyme, then zero order kinetics will apply. On the other hand, if the rate-determining step is the formation of the complex, then first order kinetics with respect to the substrate concentration will apply. That is, a substrate concentration-independent first order reaction constant is found when the K_m is high and the substrate concentration is low. If both the zero and first order equations are combined, a mathematical treatment will give the integrated form of the Michaelis-Menton equation (58) as developed by Neurath and Schwert (60). This equation has the following form:

$$k'et = 2.303\ K_m \log \frac{a}{a-x} + x$$

where x is the amount of substrate a undergoing reaction in time t, and e is the amount of enzyme. The two parts of this equation are a first order term which is predominate when

the K_m is large and the substrate concentration small and a zero order term which increases in proportion to an increase in initial substrate concentration. This equation simply states that the concentrations of free enzyme and combined enzyme at equilibrium are of the same magnitude and that the over-all rate is determined by both the rate of enzyme-substrate formation and breakdown. Thus, the integrated rate equation will bring the zero and first order effects together, and a plot of the right-hand side of the equation against time at different substrate concentrations will give straight lines having the same slope. The integrated reaction constant k' will remain constant over the entire substrate range providing higher order effects such as enzyme inactivation are not present.

A plot of the integrated Michaelis-Menton equation against time for the 0.0140M curve in Figure 5 did not give a straight line as seen in Figure 6. Since the individual effects of enzyme and substrate concentration are integrated in the mixed-order plot, the decrease in initial slope of the curve was assumed to involve higher order effects due to enzyme inactivation. This was shown to be the case as presented later in this section. Table 3 gives the initial k'e values in air calculated from the slopes of curves for four separate experiments where the substrate concentration was varied. Unfortunately, the enzyme concentration was not

Figure 6. Plot of the integrated form of the Michaelis-Menton equation for the mycelial enzyme in air. (Data taken from the 0.0140M ascorbate curve in Figure 5, K_m = 0.00115M).

a: initial amount of ascorbate, x: amount of ascorbate used in time t.

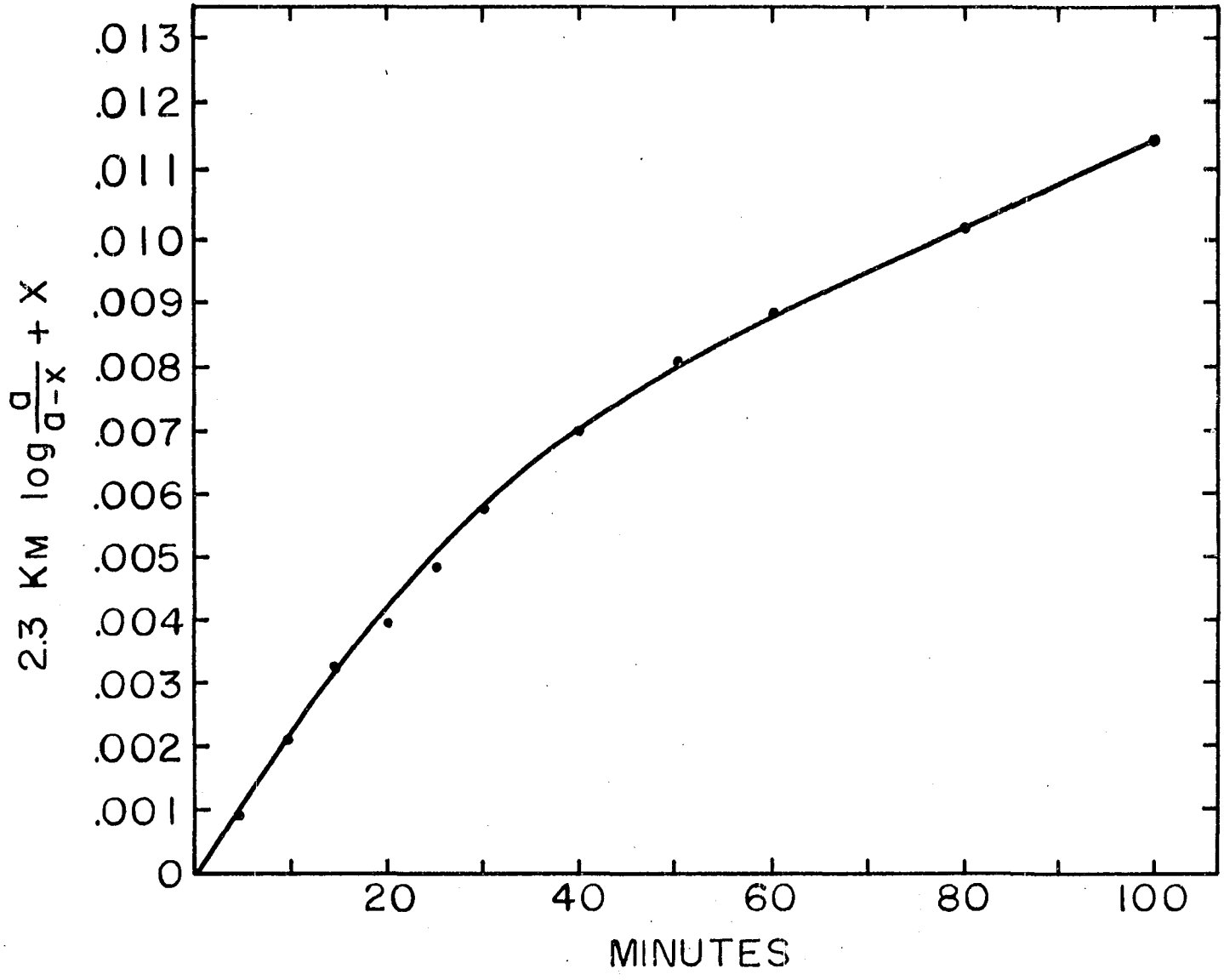

.013
.012
.011
.010
.009
.008
.007
.006
.005
.004
.003
.002
.001
0
2.3 KM log a/(a-x) + X
20
40
60
80
100
MINUTES

Table 3. Comparison of initial k'e values at varying substrate concentrations for the mycelial enzyme in air and O_2 and for the spore enzyme in air.

Date		Gas Phase	Ascorbate a (M)	Initial k'e[a] (moles/min. x 10^4)
2-11-56	Mycelium[b]	Air	0.0014	2.40
			0.0017	2.00
			0.0021	1.85
			0.0031	2.00
			0.0059	1.95
			0.0084	2.25
			0.0112	2.05
			0.0140	2.10
9-11-56	Mycelium	Air	0.0014	3.35
			0.0028	2.25
			0.0084	2.25
			0.0140	2.35
21-11-56	Mycelium	Air	0.0022	2.15
			0.0028	2.10
			0.0042	2.20
			0.0056	2.15
			0.0084	1.95
27-7-57	Mycelium	Air	0.0028	2.40
			0.0056	2.05
			0.0084	2.06
			0.0112	2.10
			0.0140	2.05
27-7-57	Mycelium	O_2	0.0028	6.60
			0.0056	5.00
			0.0084	4.80
			0.0112	4.50
			0.0140	4.40
22-12-57	Spore[c]	Air	0.0031	2.00
			0.0056	1.95
			0.0112	2.05
			0.0168	2.05
			0.0226	2.05

[a]Integrated reaction constant.

[b]Each flask contained ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate, gas phase air or pure O_2.

[c]Each flask contained ascorbate, pH 6.25, 0.034M phosphate-0.017M citrate, gas phase air.

known, thus the specific rate constant k' could not be calculated. The constant k'e includes all the individual rate constants in the over-all reaction. The k'e values were essentially constant over a 10-fold substrate range with a slight tendency towards higher initial values at the lower substrate concentrations. It would be logical to expect an increase in k'e at the slower reaction rates since the rate of change of e (enzyme concentration) would decrease at lower velocities. There was some indication of this in the shape of the integrated curves with time, however the results were scattered and did not permit accurate analysis. To allow some idea of the relative contribution of the first and zero order terms at various substrate concentrations, Table 4 provides the calculated results of the 0.0031M, 0.0084M and 0.0140M ascorbate curves in Figure 5. It can be seen that zero order effects were predominate over first order effects even at 0.0031M with the first order contribution increasing with time as was expected. At the 0.0140M level, the zero order term contributed approximately 90 percent to the initial k'e. At 0.0084M, the first-order plot (Figure 5) was "pseudo first order" apparently due to enzyme inactivation which affected the first order contribution as substrate depletion was approached.

As will be shown in the section on O_2 tension effects on the enzyme, the rate of ascorbate oxidation by the mycelial

Table 4. Data for reaction curves at 0.0031M, 0.0084M and 0.0140M ascorbate in air. (Figure 5).

a (M)	t (min.)	% a used	$2.3K_m \log \frac{a}{a-x}$ [a] ($\times 10^4$)	x (M $\times 10^4$)	k'e $\times 10^4$ [b] (moles/min.)
0.0031	5	22.9	3.10	7.10	2.04
	10	47.8	8.18	14.90	2.31
	15	66.8	12.90	20.70	2.24
	20	73.8	15.80	22.80	1.92
	25	82.0	20.40	25.40	1.83
	30	92.4	30.40	28.60	1.97
	35	96.6	38.50	29.90	1.95
	40	100.0	49.00	31.00	2.00
0.0084	5	11.0	1.35	9.40	1.91
	10	25.6	3.40	21.80	2.52
	15	34.9	4.97	29.90	2.33
	20	42.4	6.38	36.10	2.13
	25	48.4	7.66	41.50	1.96
	30	56.2	9.56	48.20	1.92
	35	61.4	11.00	52.70	1.82
	40	66.6	12.80	57.20	1.75
	45	71.8	14.80	61.60	1.70
	50	75.6	16.40	64.80	1.63
	60	80.2	18.90	68.80	1.46
	80	89.0	25.90	76.30	1.28
	100	95.0	35.00	81.30	1.16
	120	97.4	44.40	82.00	1.05
0.0140	5	5.6	0.67	7.90	1.72
	10	13.8	1.44	19.20	2.07
	15	21.0	2.70	29.30	2.14
	20	25.7	3.39	35.80	1.97
	25	31.6	4.26	43.70	1.92
	30	36.9	5.25	51.60	1.90
	35	40.6	5.95	56.80	1.80
	40	44.6	6.80	62.60	1.74
	50	51.5	8.35	72.00	1.62
	60	56.2	9.48	78.70	1.48
	80	63.6	11.60	89.20	1.26
	100	70.6	14.10	98.80	1.14
	120	75.4	16.10	106.00	1.10
	140	80.7	17.80	110.00	0.91

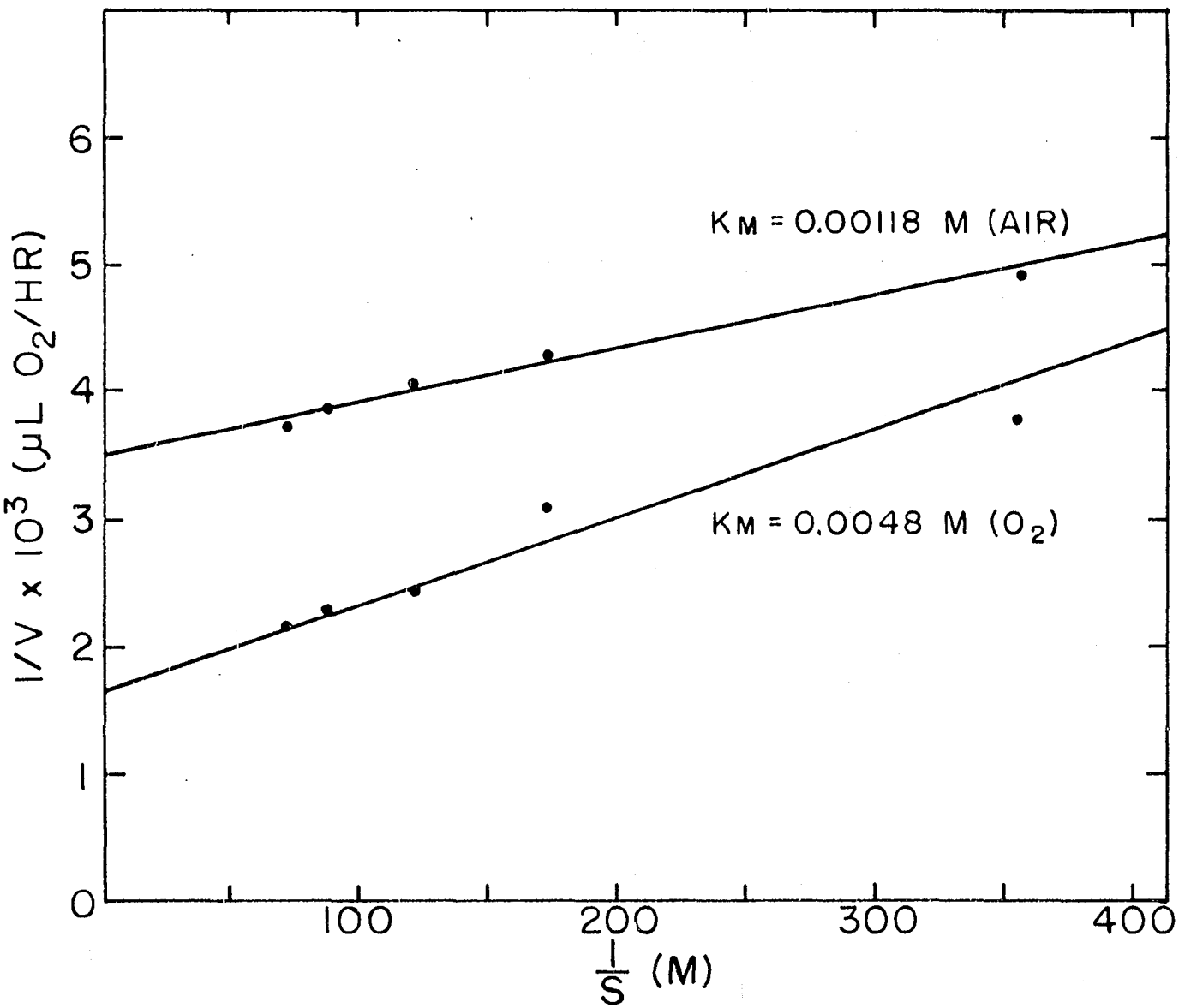
[a] a: initial concentration of ascorbate
x: amount of ascorbate used in time t.

[b] Integrated reaction constant.

enzyme increased with O_2 tension. This was expected since the molar concentration of O_2 in solution at 30° C was approximately equal to the molar O_2 affinity of the enzyme. This means, then, that as the O_2 tension increases, the rate of enzyme-substrate complex breakdown would increase, shifting the equilibrium to the formation of the complex. Thus, the K_m should be higher in O_2 than in air. This was found to be the case with the mycelial enzyme as shown in Figure 7, which is a Lineweaver-Burk (47) plot of the effect of ascorbate concentration in O_2 and air on the same enzyme preparation. The reciprocal plot gave an apparent K_m in air of 0.00118M and in 100 percent O_2 of 0.0048M. In this case, the assumption of the Michaelis-Menton theory (58) that the affinity constant is simply the equilibrium constant of the enzyme-substrate reaction does not apply since the K_m was approximately proportional to the acceptor (O_2) concentration. As Chance (8) points out for oxidative reactions where both a donor and acceptor are involved, the K_m is likely to be dependent on the acceptor concentration providing the donor concentration is sufficient. In his work with catalase and peroxidase, Chance (8) uses the bimolecular rate constant k_4 to denote the special case where the enzyme-substrate (donor) complex combines with an acceptor to form the reaction products. Similar results to those for the mycelial ascorbic acid oxidase have been obtained by Laser (46)

Figure 7. Lineweaver-Burk reciprocal plot of reaction rate of the mycelial enzyme in air and O_2 as a function of ascorbate concentration.

Reaction conditions: pH 4.5, 0.034M phosphate-0.017M citrate, gas phase air or pure O_2.


K_M = 0.00118 M (AIR)
K_M = 0.0048 M (O_2)
1/V x 10^3 (μL O_2/HR)
0
1
2
3
4
5
6
100
200
300
400
$\frac{1}{S}$ (M)

for glucose oxidase and by Slater and Bonner (71) for succinic dehydrogenase where the apparent K_m was also a function of the acceptor concentration.

The results of a kinetic analysis of the effects of ascorbate concentration in O_2 were similar to those in air except that the higher order effects due to enzyme inactivation increased with the increased reaction rates in O_2. A conventional first order plot of the rate curves in O_2 at varying ascorbate concentrations gave a series of curves which sloped downward toward the time axis. As seen in Table 5 which gives the data from 0.0028M, 0.0084M and 0.0140M ascorbate curves in O_2, the first order contribution in O_2 was greater than in air as would be expected with an increase in the K_m. The initial k'e values in O_2 were a function of the ascorbate concentration as shown in Table 3. This is in agreement with the reasoning that the rate of enzyme inactivation increases with reaction velocity and becomes more apparent in O_2 where the initial reaction rates are more affected by substrate concentration than in air. It should be mentioned here that enzyme inactivation occurred only during reaction and was not found when the enzyme and ascorbate were incubated together under pure N_2 gas or the enzyme alone under O_2 for 30 minutes before flushing with air. The enzyme was also stable to aeration for several hours at 30° C. The reaction product, dehydroascorbate,

Table 5. Data for reaction curves at 0.0028M, 0.0084M and 0.0140M ascorbate in O_2.

a (M)	t (min.)	% a used	$2.3K_m \log \frac{a}{a-x}$ [a] (x 10^4)	x (M x 10^4)	k'e x 10^4 [b] (moles/min.)
0.0028	5	37.5	22.40	10.50	6.58
	10	67.0	52.80	18.00	7.08
	15	81.2	79.00	22.80	6.80
	20	87.4	99.00	24.50	6.18
	25	100.0	198.00	28.00	9.04
0.0084	5	17.7	9.45	24.35	4.87
	10	35.4	20.90	29.80	5.07
	15	48.4	31.70	40.70	4.83
	20	56.7	39.50	47.70	4.35
	25	63.5	48.30	53.40	4.06
	30	68.6	56.00	57.80	3.79
	40	76.0	68.20	64.00	3.30
	50	80.0	77.00	67.40	2.87
	60	82.8	83.50	69.50	2.55
	70	85.3	91.20	71.60	2.33
	80	88.0	101.00	73.80	2.19
	100	90.5	112.00	76.00	1.88
	120	92.2	119.00	77.00	1.63
0.0140	5	10.6	5.40	14.90	4.05
	10	24.7	12.40	34.80	4.72
	15	34.7	17.60	48.50	4.40
	20	42.5	26.50	59.50	4.30
	25	47.5	30.90	66.40	3.88
	30	52.4	35.30	73.40	3.62
	40	57.8	40.80	81.00	3.05
	50	63.1	47.50	88.00	2.71
	60	66.0	52.00	92.00	2.40
	70	68.4	55.20	96.00	2.17
	80	70.6	58.60	99.00	1.98
	100	74.4	65.20	104.00	1.69
	120	76.3	68.60	107.00	1.46

[a] a: initial concentration of ascorbate
x: amount of ascorbate used in time t.

[b] Integrated reaction constant.

at 0.01M had no effect on the enzyme. A plot of the integrated equation for the enzyme in O_2 and with 0.0140M ascorbate is given in Figure 8. The initial slope (k'e) was greater than in air while the curve sloped off more rapidly than in air due to increased enzyme inactivation.

As presented in the next section in this thesis, the mycelial enzyme was found to be surface-localized, and it would oxidize ascorbate at rates comparable to the extracted enzyme. It became of interest to see whether the intact enzyme had the same kinetic properties as the solubilized enzyme, particularly since Honda (37) found that the surface-localized ascorbic acid oxidase in barley roots failed to show reaction inactivation while the extracted oxidase did. As shown for a typical experiment in Figure 9, the rate curve fell off gradually with time even at high substrate concentrations. Dehydroascorbate was apparently not oxidized further since no excess O_2-CO_2 gas exchange occurred over the endogenous rate. The integrated Michaelis-Menton plot as shown in Figure 10 gave a curve similar in shape to the one in air, indicating reaction inactivation.

Since the mixed-order plots previously presented were not linear, the possibility of kinetically analyzing the inactivation of the mycelial enzyme was considered. Figure 11 is a semilog plot of the k'e values from the mixed order curve for O_2 (curve 1), air (curve 2) and the intact enzyme

Figure 8. Plot of the integrated form of the Michaelis-Menton equation for the mycelial enzyme in O_2. (K_m = 0.0048M).

Reaction conditions: 0.0140M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate, gas phase pure O_2. a: initial amount of ascorbate, x: amount of ascorbate used in time t.

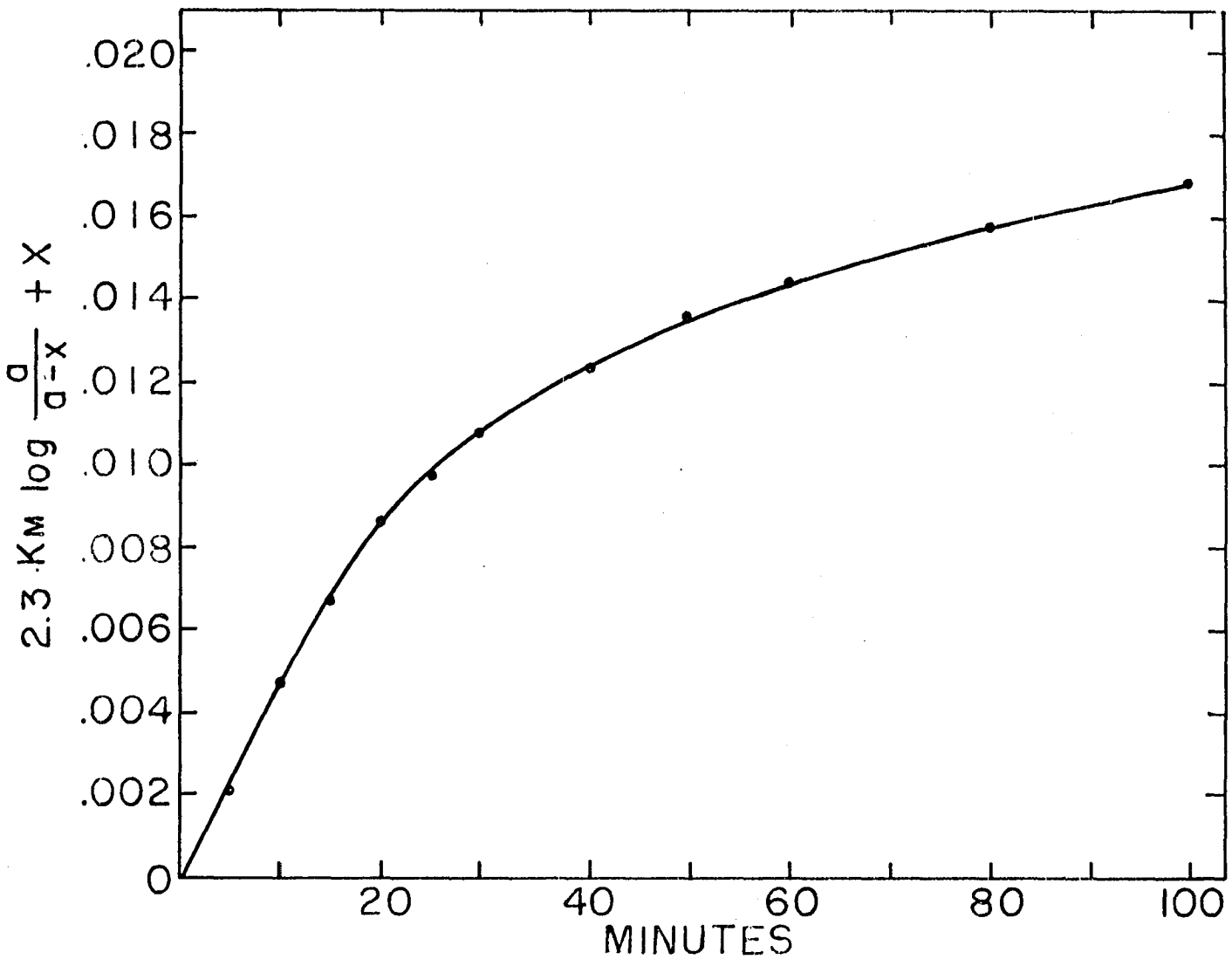

.020
.018
.016
.014
.012
.010
.008
.006
.004
.002
0
2.3 Km log a/a-x + X
20
40
60
80
100
MINUTES

Figure 9. Reaction curve for the oxidation of ascorbate by the intact enzyme.

Reaction conditions: 5.6 mg. dry weight of mycelium, 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate, Theoretical stoichiometry was 256 microliters of O_2.

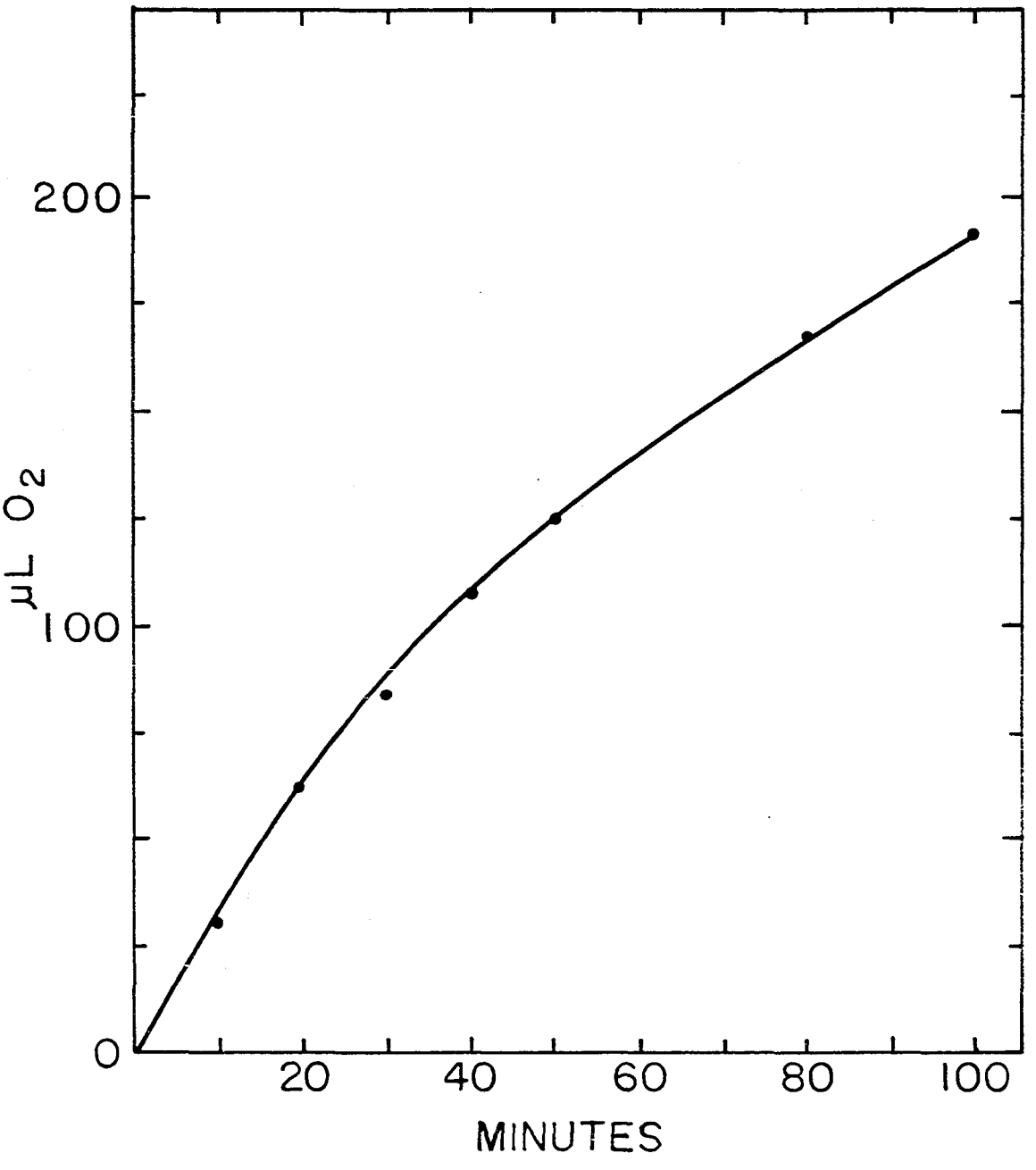

200
100
0
µL O2
20
40
60
80
100
MINUTES

Figure 10. Plot of the integrated form of the Michaelis-Menton equation for the intact enzyme. (Data taken from the reaction curve in Figure 9, K_m = 0.00115M.)

a: initial amount of ascorbate, x: amount of ascorbate used in time t.

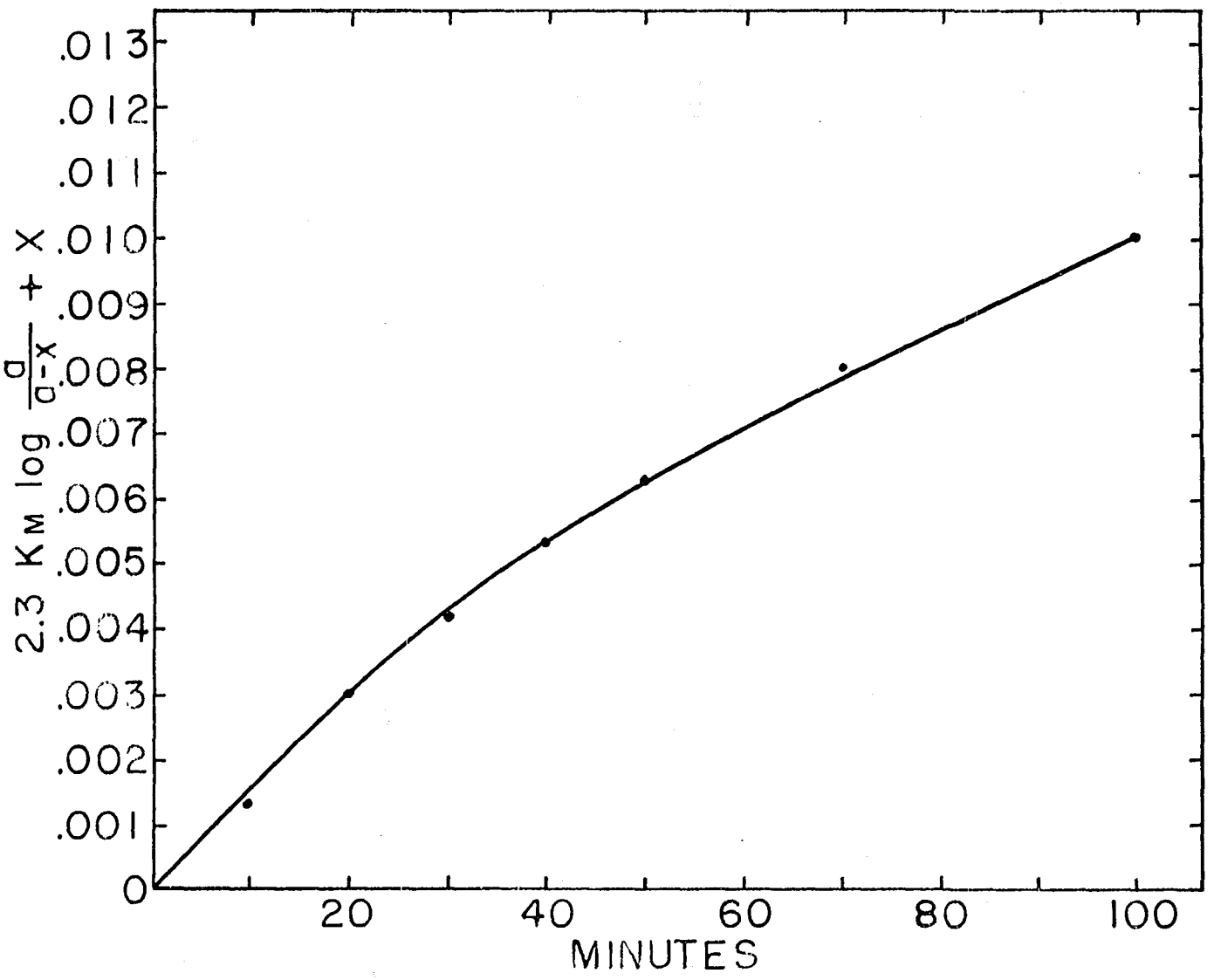
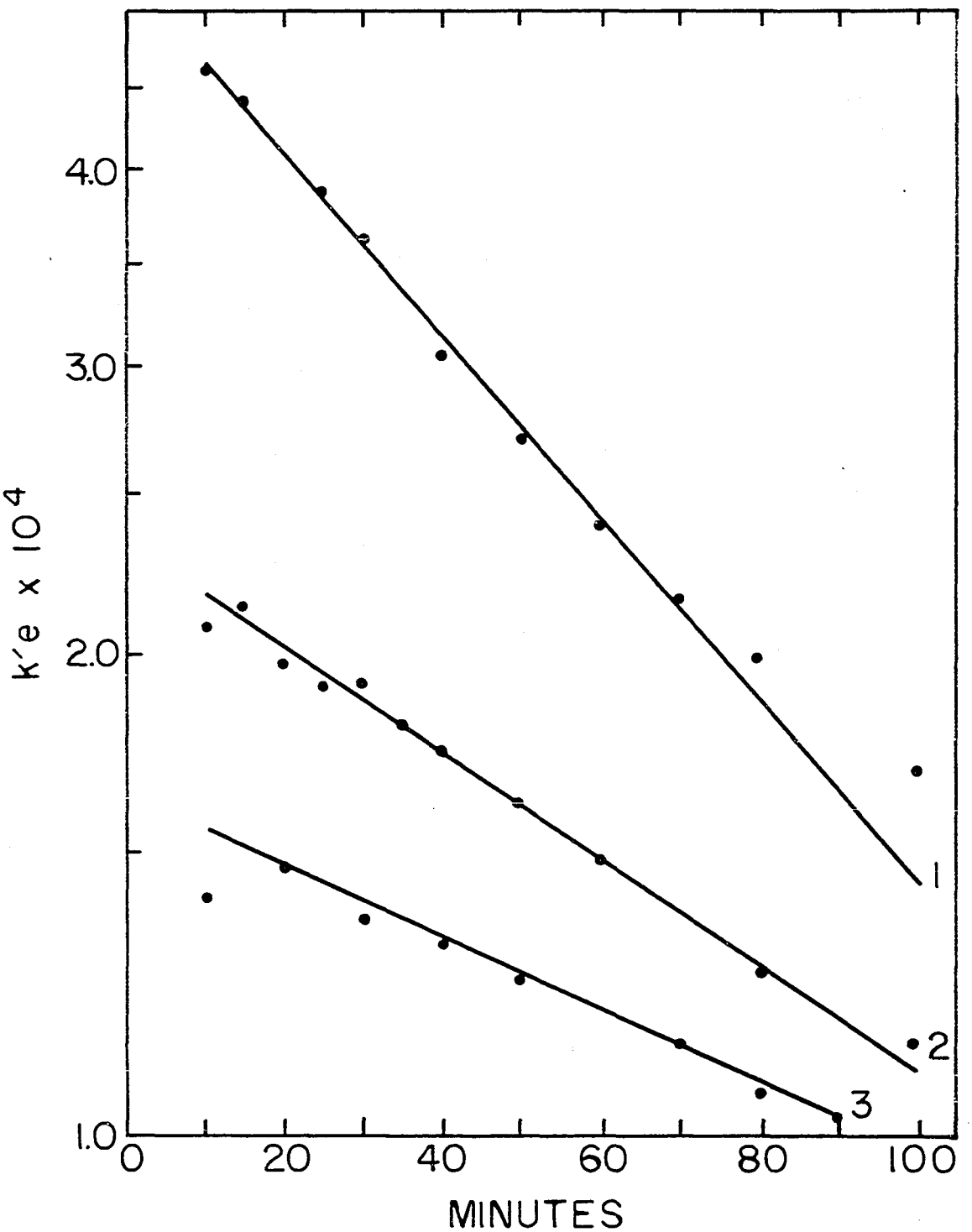

.013
.012
.011
.010
.009
.008
.007
.006
.005
.004
.003
.002
.001
0
2.3 Km log $\frac{a}{a-x}$ + X
20
40
60
80
100
MINUTES

Figure 11. Semilog plot of calculated k'e values.

Curve 1 - Enzyme extract in O_2. Data taken from the curve in Figure 8.
Curve 2 - Enzyme extract in air. Data taken from the curve in Figure 6.
Curve 3 - Intact enzyme in air. Data taken from the curve in Figure 10.


4.0
3.0
2.0
1.0
k'e x 10⁴
0
20
40
60
80
100
MINUTES
1
2
3

(curve 3). These are taken from the curves in Figures 8, 6, and 10, respectively. The linearity of such plots indicated that reaction inactivation was first order with respect to the enzyme concentration. Since the reaction velocity and hence k'e was increased in O_2, the slope of the O_2 curve was larger than the slope of the curve in air. Table 6 compares the slopes of the log k'e values for several experiments with the intact enzyme in air and the extracted enzyme in O_2 and air. The rate of reaction inactivation was apparently not constant since the slopes varied among different enzyme preparations. Unlike the Cu

Table 6. Comparison of slopes of log k'e values for the extracted mycelial oxidase in O_2 and air and the intact mycelial oxidase in air.

Enzyme	Gas Phase	Experiment	Ascorbate (M)	Slope log k'e
Extract	Air	2-11-56	0.0140	-0.0034
		9-11-56	0.0140	-0.0040
		21-11-56	0.0112	-0.0029
		22-5-57	0.0140	-0.0028
		27-7-57	0.0140	-0.0023
		18-4-58	0.0140	-0.0040
Extract	O_2	21-6-57	0.0112	-0.0077
		27-7-57	0.0140	-0.0060
		25-2-58	0.0168	-0.0070
		6-12-58	0.0140	-0.0059
Intact	Air	1-10-57	0.0140	-0.0025
		14-12-57	0.0112	-0.0023
		18-12-57	0.0112	-0.0022
		17- 9-58	0.0140	-0.0025

enzyme (64) a 5 percent solution of proteins such as gelatin bovine albumin and cytochrome c did not activate or protect the system. Cysteine, EDTA, diethyldithiocarbamate, cyanide and aluminum ions also were ineffective in activation or protection although they had marked effects at low concentrations on the Cu oxidase (29).

The kinetics of the spore enzyme in air proved to be the same as for the mycelial enzyme. As shown in Figure 12, the same general shape of the mixed-order curve was found; and, as seen in Figure 13, a semilog plot of the k'e values from this curve gave a first order rate of enzyme inactivation. In addition, the conventional first order plot yielded non-linear curves which increased in slope with decreasing substrate concentration and which could be analyzed using the integrated Michaelis-Menton equation. The initial k'e values obtained at different substrate concentrations are given in Table 3. These values, like those for the mycelial enzyme in air, appeared to be independent of the initial substrate concentration. No data are available for the spore enzyme reaction in O_2.

In brief summary, the kinetics of the spore and mycelial enzymes are consistent with the integrated form of the Michaelis-Menton equation which has a first order term predominating when all of the enzyme is not saturated with substrate and a zero order term which applies to the

Figure 12. Plot of the integrated form of the Michaelis-Menton equation for the spore enzyme. (K_m = 0.00175M).

Reaction conditions: 0.0168M ascorbate, pH 6.2, 0.034M phosphate-0.017M citrate. a: initial amount of ascorbate, x: amount of ascorbate used in time t.

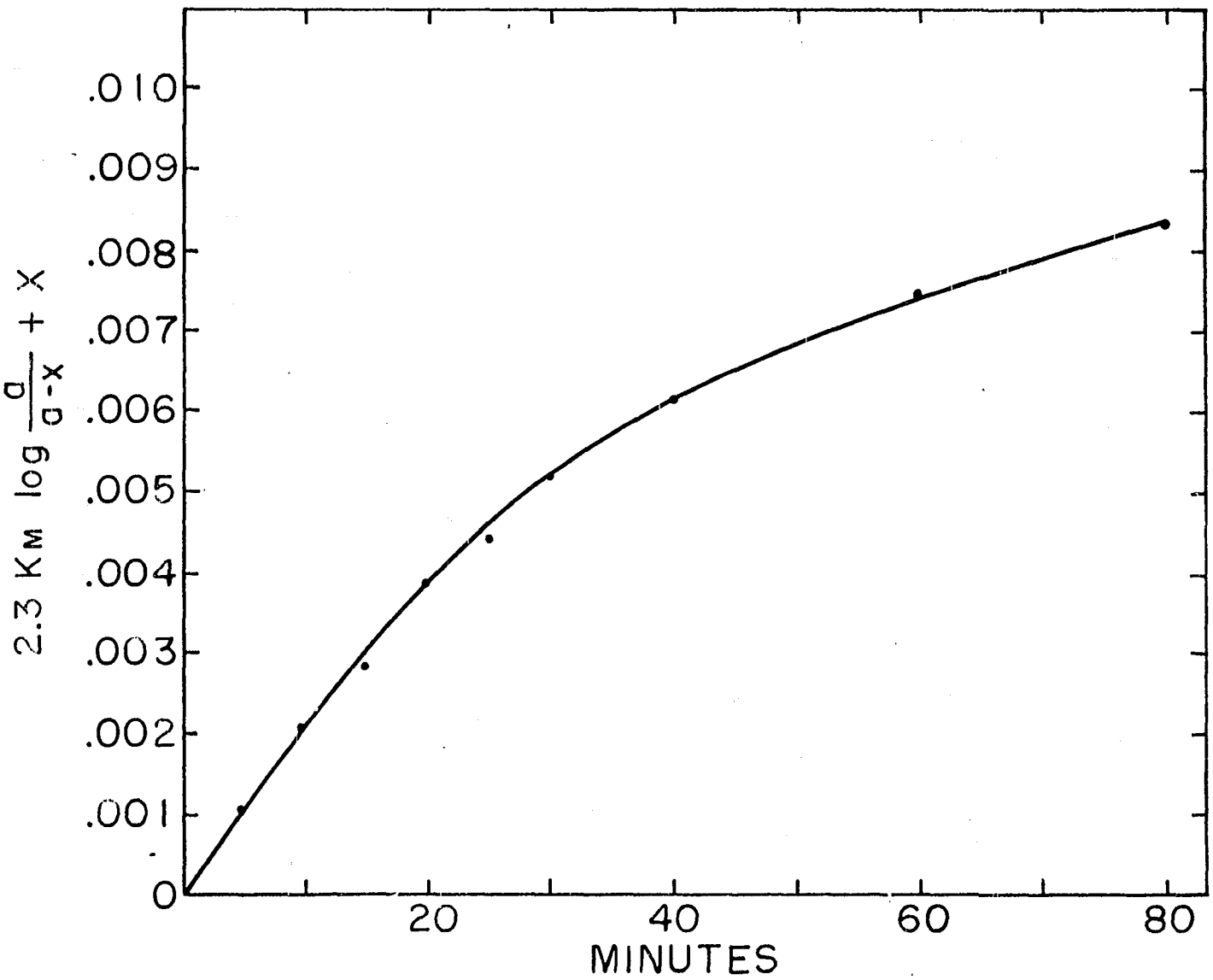
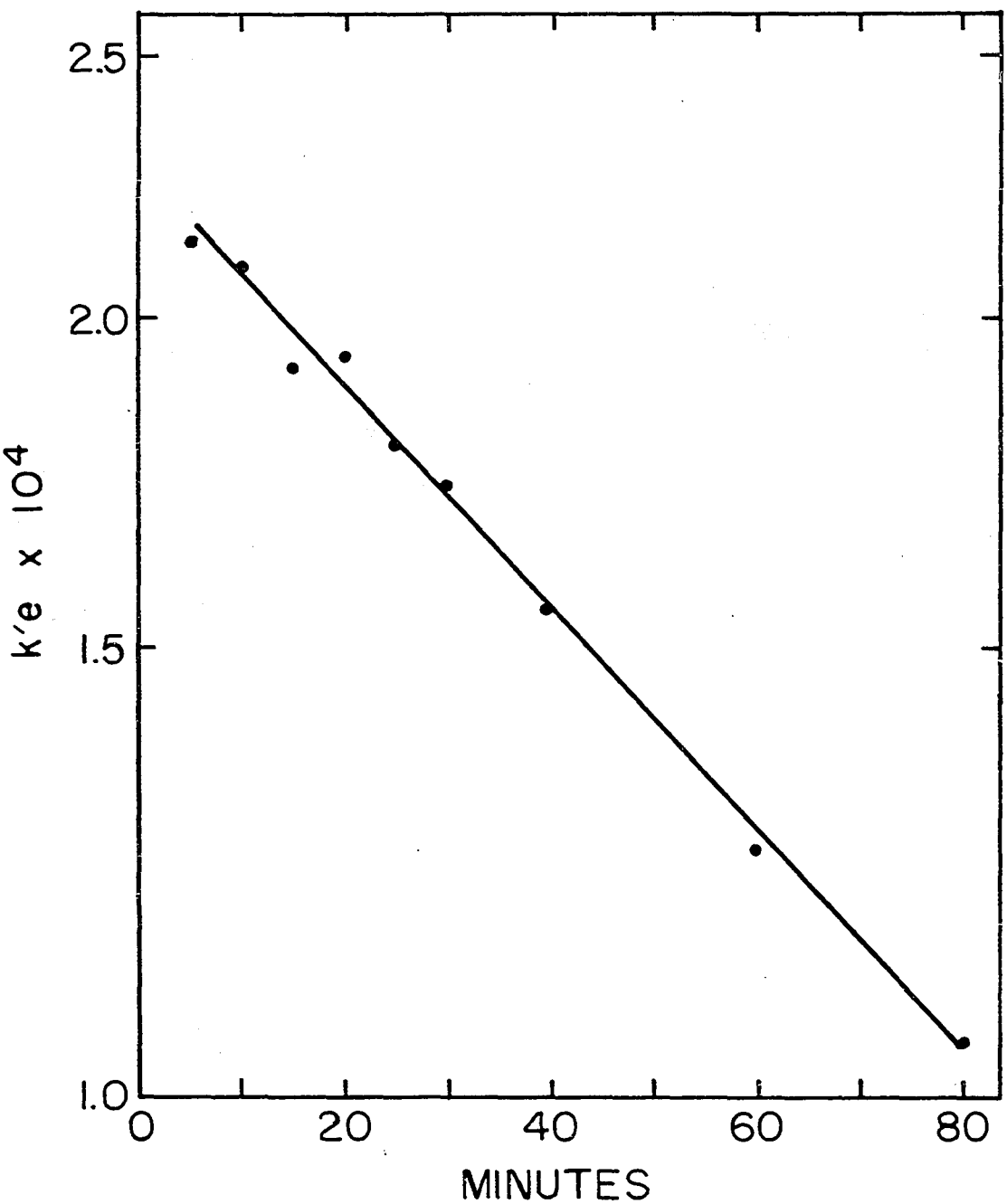

.010
.009
.008
.007
.006
.005
.004
.003
.002
.001
0
2.3 Km log a/a-x + X
20
40
60
80
MINUTES

Figure 13. Semilog plot of the calculated k'e values for the spore enzyme. (Data taken from the curve in Figure 12.)


2.5
2.0
1.5
1.0
k'e x 10⁴
0
20
40
60
80
MINUTES

situation when the maximal velocity has been attained. The zero order contribution was more important in the case of the atypical enzymes since the K_m was relatively small. The fact that the integrated equation takes out the effect of substrate concentration on k' showed that the change in k'e with time was due to a decrease in the concentration of active enzyme. As would be expected, the rate of enzyme inactivation increased with reaction velocity.

Effect of Inhibitors on the Mycelial Enzyme and Tests for a Prosthetic Group

The typical ascorbic acid oxidase is a Cu-protein which is characteristically inhibited by such Cu-binding agents as diethyldithiocarbamate, 8-hydroxyquinoline, phenylthiourea, ethyl xanthate and cyanide. It became of interest, then, to see how the atypical enzyme responded to these and several other types of inhibitors in an effort to obtain evidence for a metal prosthetic group and on the general nature of the enzyme. The inhibitors used are known to inhibit metal, sulfhydryl or flavoenzymes and are presented in this order in Table 7. The results in Table 7 are expressed as average percentage initial inhibition or stimulation (negative values) over the controls. More complete results upon which these averages were based are presented in Table 19 in the Appendix. The range of inhibition is given for the experiments with 8-hydroxyquinoline, diethyldithiocarbamate,

Table 7. Inhibitor response of the mycelial enzyme. (Complete results in Table 19 in Appendix).

No. of Expts.	Inhibitor[a]	Conc. (M)	Average inhibition[b] (%)	Range
2	Cyanide	0.01	35	
2	Cyanide	0.001	20	
2	Azide	0.01	31	
2	Azide	0.001	33	
2	CO-dark	19:1[c]	0	
2	CO-light	19:1	0	
3	8-Hydroxquinoline	0.01	14	-7 to 34
4	Diethyldithiocarbamate	0.01	-4	-26 to 14
2	Diethyldithiocarbamate	0.001	-18	
1	Diethyldithiocarbamate	0.00001	0	
2	Ethyl xanthate	0.005	30	
1	Ethyl xanthate	0.001	4	
4	Phenylthiourea	0.01	-4	-20 to 16
2	Thiourea	0.01	8	
1	Thioglycolate	0.01	18	
1	BAL	0.001	5	
1	o-Phenanthroline	0.001	0	
1	2,2'-Bipyridyl	0.001	0	
3	EDTA	0.001	16	
2	EDTA	0.0001	21	
2	EDTA	0.000001	11	
2	o-Iodosobenzoate	0.01	-2	
3	Iodoacetate	0.01	-15	-41 to 6
2	Iodoacetamide	0.01	7	
2	Phenylarsenoxide	0.01	1	
4	p-Chloromercuribenzoate	0.005[d]	-22	-53 to 0
1	Arsenite	0.01	-3	
1	Ferricyanide	0.001	-8	
3	Riboflavin	0.001	0	
2	Isoriboflavin	0.001	0	
3	Atabrine	0.001	0	
3	FMN	0.001	0	
1	FAD	0.00012	0	

[a]Standard reaction mixture contained 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate. No preliminary incubation with inhibitor.

[b]Negative values indicate stimulation.

[c]95% CO: 5% O_2 at 1 atm. Control, 95% N_2: 5% O_2.

[d]Calculated molarity. Actual concentration in solution was less than 0.005M.

phenylthiourea, iodoacetate and parachloromercuribenzoate where considerable variation in response was found.

It can be seen from the data that the enzyme was relatively resistant to high concentrations (0.01M) of metal oxidase inhibitors. Cyanide and azide inhibited only 30 percent at 0.01M and were about as effective at 0.001M. Carbon monoxide (19:1) had no effect in light or dark. While cyanide, azide, 8-hydroxyquinoline, diethyldithiocarbamate and ethyl xanthate inhibit the Cu enzyme at 0.01M (22,50), they had no comparable effect on this enzyme. Diethyldithiocarbamate at 0.001M actually stimulated the enzyme, although in constrast to Ward's system (81), catalytic amounts (10^{-5}M) failed to stimulate. Thiourea, thioglycolate and BAL which have also been used to inhibit Cu oxidases were only weakly inhibitory. EDTA inhibited 16 percent at 0.001M and had noticeable effects on the enzyme as low as 10^{-6}M. Ortho-phenanthroline and bipyridyl did not inhibit in single tests at 0.01M.

The enzyme had no apparent need for essential sulfhydryl groups since high concentrations of thiol reagents did not inhibit but in several cases were even stimulatory. Iodoacetate and parachloromercuribenzoate stimulated by 15 and 22 percent, respectively. The rest of the thiol reagents (o-iodosobenzoate, iodoacetamide, arsenite, phenylarsenoxide and ferricyanide) had little or no effect.

Iodoacetate and parachloromercuribenzoate might stimulate the enzyme by reacting with inhibiting sulfhydryl groups near the reactive site or soluble thiol compounds in the crude extract. The lack of marked inhibition by thiourea and BAL, however, would tend to discount inhibition by endogenous thiols. The inhibitor data definitely indicated that Cu was not involved in catalysis as it is for the typical enzyme. The probability of Fe being the prosthetic group seems unlikely considering the results with cyanide, azide and CO. The participation of cytochrome oxidase is also negated by the lack of strong inhibition with these compounds. It must be mentioned, however, that the possibility of another metal still remains since several of the metal binders (cyanide, azide, EDTA, and ethyl xanthate) had some inhibitory effects. Preliminary results with Mo, Mn and Mg salts showed inhibition by these ions at 0.001M.

Another possibility considered was that the enzyme is a flavoprotein. Since dye coupling experiments were not feasible with ascorbate as the substrate, several flavin enzyme inhibitors (67) were tested. Riboflavin, isoriboflavin and atabrin at 0.001M had no effect on the reaction suggesting that the enzyme was not a flavoprotein. Neither FAD or FMN stimulated the crude enzyme showing that any flavin group was not dissociated during enzyme extraction. Unfortunately, no highly specific inhibitors of flavoenzymes

are known so the results of these tests do not provide unequivocal evidence against a flavin prosthetic group. Whether the enzyme has a flavin prosthetic group and which metal if any, is involved, awaits purification of the protein.

The same inhibitors were tested on the soluble and insoluble fractions to determine if there were any differences in response. Table 8 gives the significant differences observed in inhibitor response between the two fractions. To illustrate the variation found, the results are given for individual experiments on fractions separated from the same enzyme preparation. Generally, the response to most inhibitors was quite similar for both fractions. However, the soluble fraction was less sensitive to cyanide and azide but considerably more sensitive to 8-hydroxyquinoline and phenylthiourea. Diethyldithiocarbamate possibly had a greater stimulatory effect on the insoluble fraction. What these differences mean is not clear. Partial denaturation and exposure of the active site to inhibitor may explain the increased inhibition effect with the insoluble fraction.

Dialysis of several metal enzymes and metalloflavoproteins against cyanide has been found to effect the removal of the metal prosthetic group. The resulting inhibition can be reversed by re-adding the metal ion. This has been found with cucumber ascorbic acid oxidase (57), polyphenol

Table 8. Differences between the soluble and insoluble fractions in response to inhibitors.

Inhibitor	Conc. (M)	Expt.[a]	Inhibition (%)[b] Insol.	Sol.
Cyanide	0.001	2-12-56	82	-2
		14-3-57	---	3
		25-3-57	22	---
		1-4-57	30	0
Azide	0.001	2-12-56	83	-3
		1-4-57	38	-4
8-Hydroxyquinoline	0.01	1-12-56	6	47
		1-4-57	6	35
Phenylthiourea	0.01	1-12-56	0	40
		2-4-57	6	17
Diethyldithiocarbamate	0.001	1-12-56	-30	-18
		1-4-57	-27	-10

[a]All flasks contained 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate. No preliminary incubation with inhibitor.

[b]Negative values indicate stimulation.

oxidase (45) and nitrate reductase (62), a molybdo-flavo-protein. It was decided that this technique might provide additional evidence for a metal prosthetic group, particularly Cu, in the atypical oxidase. Table 9 gives the results of two experiments in which cupric and ferric ions were added to the cyanide-dialysed soluble fraction. Undialyzed enzyme stored at 8° C and pH 4.5 was used as an activity control with each successive dialysis at 8° C. The cyanide inhibition after dialysis against 0.01M cyanide

Table 9. Cyanide dialysis and metal ion restoration experiments.

Successive treatments[a]	Initial rate (ul. O_2/hr.)		Activity[b] (%)
	Dialysed	Control	
Experiment 1			
1. Untreated soluble fraction	---	300	100
2. Dialysis against 0.01M KCN	186	276	67
3. CN removal by dialysis	180	246	73
4. Addition of Cu (5×10^{-5}M) as $CuSO_4$ and dialysis	132	231	57
Experiment 2			
1. Untreated soluble fraction	---	240	100
2. Dialysis against 0.01M KCN	138	202	68
3. CN removal by dialysis	124	174	71
4. Addition of Fe (1×10^{-4}M) as $FeCl_3$	124	174	71

[a]All flasks contained 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate.

[b]Percent of non-dialysed control stored at 8° C.

for 36 hours was about 30 percent and was not significantly reversed after cyanide removal by dialysis. Neither ionic Cu or Fe added in excess were capable of restoring any activity, again suggesting that these metals were not involved. Preliminary results showed the same effects of cyanide-dialysis and metal restoration on the insoluble fraction. Dialysis of the enzyme against cold, pH 4.5, 0.01M phosphate-0.005M citrate buffer for 18 hours also failed to remove any dialysable cofactor (quinones, etc.) or to affect the activity.

Localization and Physiological Role of the Mycelial Enzyme

Localization

Ascorbic acid oxidase had been considered a soluble oxidase until evidence was presented to show that it could be localized on some higher plant cell walls (37,61). Since Mandels (51) found that the spore enzyme also was surface-localized, it became of interest to see if the mycelial enzyme was similarly associated with the mycelium surface. The possible mediation of surface-localized cytochrome oxidase in ascorbate oxidation was rejected since hydroquinone (4 mg.) plus cytochrome c (2 mg.) was not oxidized by the mycelium. Furthermore, with added ascorbate, 0.001M cyanide had no effect on O_2 uptake and cytochrome c (2 mg.) did not stimulate ascorbate oxidation by the intact mycelium.

Since the enzyme was so readily released from the mycelium during grinding in phosphate-citrate buffer or water, the use of wall fragments to determine the enzyme distribution would be open to considerable error. Consequently two other methods were used to provide evidence for surface localization of the oxidase. One of the methods which Mandels (51) used to demonstrate ascorbic acid oxidase association with the spore surface of *M. verrucaria* was to show that the pH effect on the enzyme *in vivo* was identical with the pH effect on the solubilized enzyme. This method rested on the

assumption that, unlike the cell surface, the cytoplasmic pH was not appreciably affected by the external pH. A comparison of Figures 1 and 14 shows that pH had the same effect on the mycelial enzyme activity *in vivo* as *in vitro*. The internal pH of the cell obviously was not radically altered since the endogenous respiration rate remained fairly constant. This observed pH-activity relationship supported a surface localization of the enzyme. The difference between the endogenous respiration rate and the rate with added ascorbate is given as net oxidase activity per mg. dry weight of mycelium per hour (Q_{O_2}). The endogenous respiration rate was measured at the indicated pH's for 20 minutes before adding ascorbate.

Further evidence of surface localization was obtained by determining the effect of short-term acid treatments on the intact enzyme and on the endogenous respiration. Table 10 shows that a 0.5 to 3.0 minute exposure of the mycelium to 0.05N HCl (pH 1.3) or a 5 minute exposure to 0.01N HCl (pH 2) completely inactivated the oxidase without significantly changing the rate of endogenous respiration. These results were the same whether the mycelium was previously starved or unstarved. The acid inactivation was pH irreversible as in the case of the extracted enzyme. These data also agreed with a surface localization of the enzyme since it was unlikely that acid inactivation of the enzyme could

Figure 14. Effect of pH on mycelial respiration and on the activity of the intact ascorbic acid oxidase.

Reaction conditions: 0.0140M ascorbate, 0.034M phosphate-0.017M citrate, 0.0005M Mg, 0.2 ml. KOH.

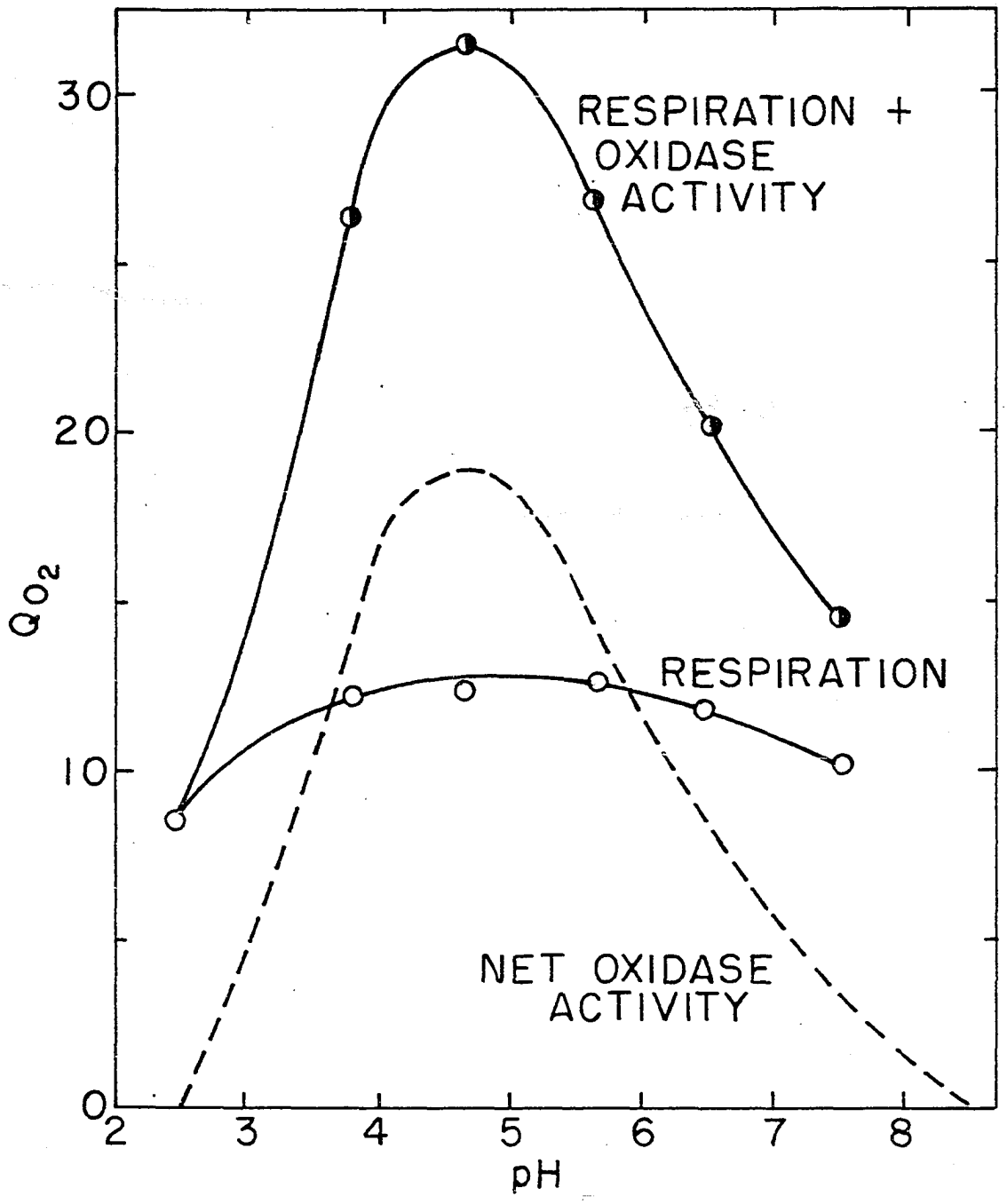

30
20
10
0
Q_{O_2}
RESPIRATION +
OXIDASE
ACTIVITY
RESPIRATION
NET OXIDASE
ACTIVITY
2
3
4
5
6
7
8
pH

Table 10. Acid inactivation of the surface-localized oxidase.

Mycelium	Treatment[a]	Oxidative activity (ul.O_2/hr./mg. dry wt.)	
		Oxidase	Respiration
Starved	Control	18.1	16.8
	0.05N HCl for 0.5 min.	1.0	16.1
Unstarved	Control	47.3	34.7
	0.05N HCl for 3.0 min.	0.0	31.8
Starved	Control	15.6	13.8
	0.01N HCl for 5.0 min.	0.0	12.6

[a]Each flask contained mycelium, 0.0100M ascorbate, pH 4.5, 0.032M phosphate-0.016M citrate, 0.004M Mg, 0.2 ml. KOH in centre well.

occur within the cell without drastically affecting the cytochrome respiratory system. In addition, no active ascorbic acid oxidase could be extracted at pH 6.2 from mycelium exposed to 0.01N HCl for 5 minutes, thus providing evidence for complete surface localization. A control run at the same time with an equal weight of untreated mycelium had an O_2 uptake of 102 microliters per hour.

Respiratory function

Since ascorbic acid oxidase has been implicated in terminal oxidation in plant tissues by numerous investigators (5,54), the question arose as to whether the atypical oxidase in Myrothecium mycelium had any role in oxidative respiration. Therefore, several experimental approaches were used to

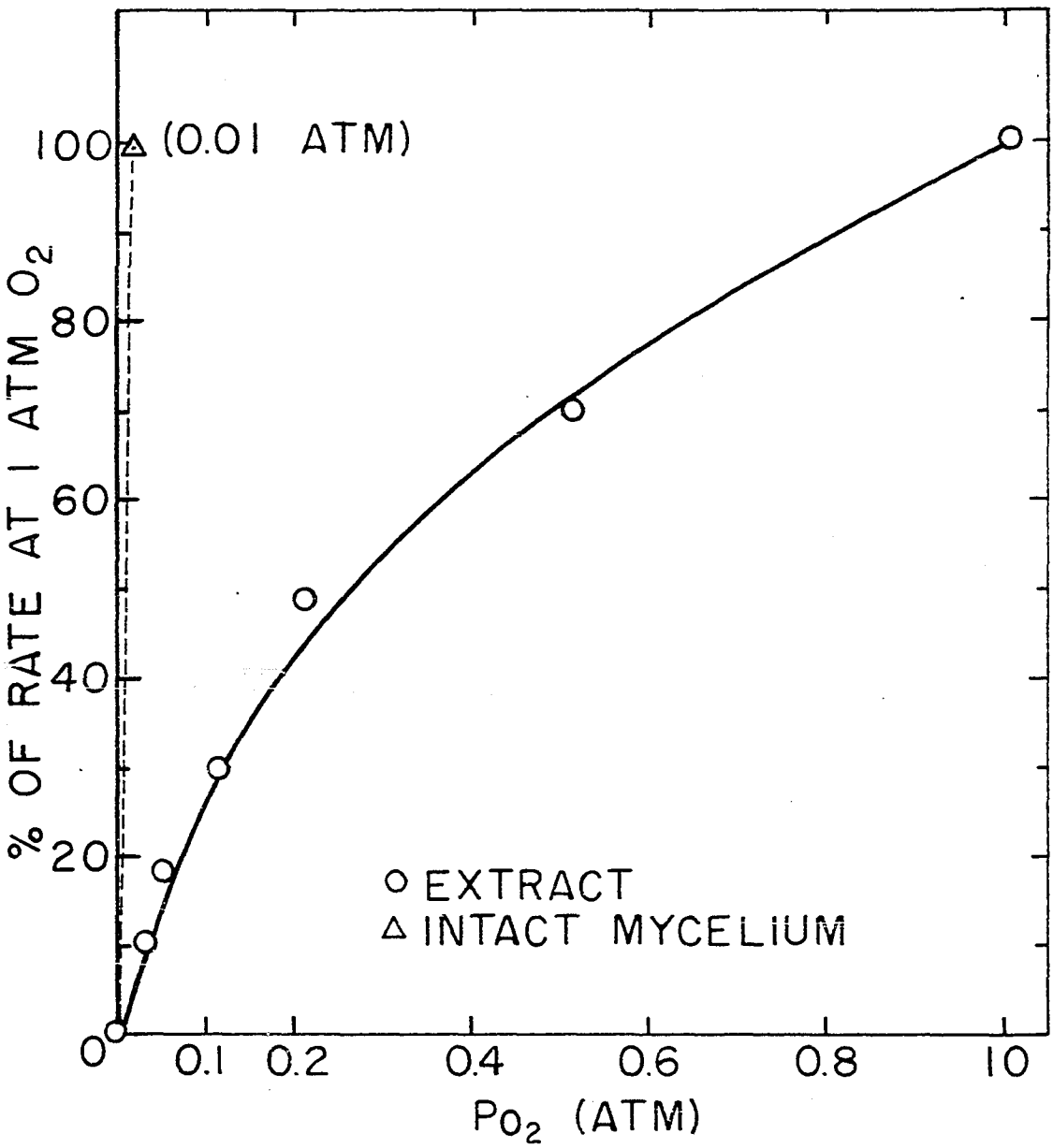
obtain quantitative evidence for the oxidase functioning in terminal respiration. The first approach was to compare the O_2 affinity of the extracted ascorbic acid oxidase with the O_2 affinity of the respiration of intact mycelium. Other workers (23,40,79,84), have employed this technique to determine the active terminal oxidase in plant tissues on the assumption that a tissue with a high affinity for O_2, compared with ascorbic acid oxidase, must depend on cytochrome oxidase. The second approach was to selectively inhibit the cytochrome chain with several specific inhibitors (SN 5949 and antimycin a) to see if any respiration remained which might be due to ascorbic acid oxidase activity.

The rate-limiting factor in the oxidation of ascorbate by the mycelial oxidase was the concentration of O_2 in the liquid phase. This dependence of oxidation rate on O_2 tension is shown for a typical experiment in Figure 15. As seen from the curve for the enzyme, the rate in air (0.2 atmospheres of O_2) was approximately 44 percent of the rate at 1.0 atmosphere of O_2. The low oxidation rates at low O_2 tensions were not caused by a slow rate of O_2 diffusion into the liquid phase since with the enzyme concentration and the shaking rate (130 oscillations per minute) used, the amount of enzyme could be increased and still allow an increase in the rate of O_2 uptake. Since the O_2 diffusion

Figure 15. Effect of O_2 tension on the initial rate of ascorbate oxidation by the extracted oxidase and on the respiration of the intact mycelium.

Reaction conditions for extracted oxidase: 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate. Each flask was gassed with 2 litres of gas mixture at 1 atmosphere.

Reaction conditions for respiration: mycelium exposed to moist gas phase, 0.3 ml. water plus paper wick in side arm, 0.2 ml. KOH. Each flask was gassed with 2 litres of gas mixture at 1 atmosphere.


100
80
60
40
20
0
(0.01 ATM)
% OF RATE AT 1 ATM O_2
EXTRACT
INTACT MYCELIUM
0.1
0.2
0.4
0.6
0.8
1.0
P_{O_2} (ATM)

was not limiting under these conditions, the calculated pO_2^{50} affinity value (partial pressure of O_2 in atmospheres needed for half-maximal velocity) of 0.254 atmospheres showed that the oxidase had a low affinity for O_2 compared with cytochrome oxidase (Table 11). The affinity value was calculated from a reciprocal plot of the initial reaction velocity against the pressure of O_2 in atmospheres (Figure 16). The linearity of the plot indicated that the O_2 tension effects on the enzyme agreed with Michaelis-Menton kinetics (58). The rate observed in air was of the magnitude expected since the concentration of O_2 in water at 30° C was 2.4×10^{-4}M and the calculated affinity constant (K_m) was 3.0×10^{-4}M. Table 11 compares the pO_2^{50} and the K_m (as micromolar O_2) for several extracted oxidase enzymes. The value of 301 micromolar for the mycelial ascorbic acid oxidase was far higher than the value of 4.5 micromolar for cytochrome oxidase in tobacco root particles or the DPNH-flavin oxidase in S. faecalis (2.1 micromolar). Interestingly, the mycelial enzyme had an O_2 affinity about 30 percent higher than that of the spore oxidase as calculated from Mandels' (52) data. The apparent O_2 affinity of the mycelial oxidase placed it close to the typical ascorbic acid oxidases (164-372 micromolar O_2) in this respect but not in the range of a flavin oxidase such as glucose oxidase which had 3 times the affinity for O_2. Sisler and Evans (69) recently

Table 11. Comparison of O_2 affinities of oxidases and respiration of *M. verrucaria* with those of other organisms.

	Source[a]	Temp. (°C)	pO_2^{50}[b] (atm.)	K_m[c] (uM)	Ref.
		Oxidases			
Cyt. c	heart muscle	25	0.0013	2.0	(7)
DPNH	*S. faecalis*	25	0.0017	2.1	(63)
Cyt. c	tobacco root	25	0.0036	4.5	(69)
Phenol	tobacco root	25	0.0118	15.0	(69)
Cyt. c	*M. verr.* mycelium	30	<0.01	<12.0	(15)
Amino acid	pig kidney	38	0.047	50.0	(46)
Glucose	*P. notatum*	38	0.094	100.0	(46)
Ascorbic acid	pea internode	25	0.13	164.0	(23)
Ascorbic acid	Bios Lab. prep.	25	0.16	206.0	(79)
Ascorbic acid	*M. verr.* mycelium	30	0.254	301.0	—
Ascorbic acid	squash	30	0.32	372.0	(52)
Ascorbic acid	*M. verr.* spores	30	0.37	431.0	(52)
		Respiration			
Aerobacter		19	0.000022	0.031	(48)
Rat liver mitochondria		25	0.00057	0.8	(1)
Baker's yeast		20	0.00065	0.9	(48)
Kidney mitochondria		25	0.0013	1.6	(9)
S. faecalis		25	0.0017	2.1	(63)
Aroid spadix		25	0.002	3.0	(84)
Potato tuber slices		25	0.002	3.0	(79)
M. verr. mycelium[d]		30	<0.005	<6.0	—
Torula yeast		10	0.0094	16.0	(80)
Pea internode		25	0.031	38.0	(23)
M. verr. mycelium[e]		30	0.10	117.0	(14)

[a]Affinity values except for mycelial oxidase and mycelial respiration were taken from the literature or calculated from published data.

[b]pO_2^{50}: partial pressure of O_2 in atmospheres required for half-maximal velocity.

[c]Expressed as micromolar O_2 and calculated from the equation:

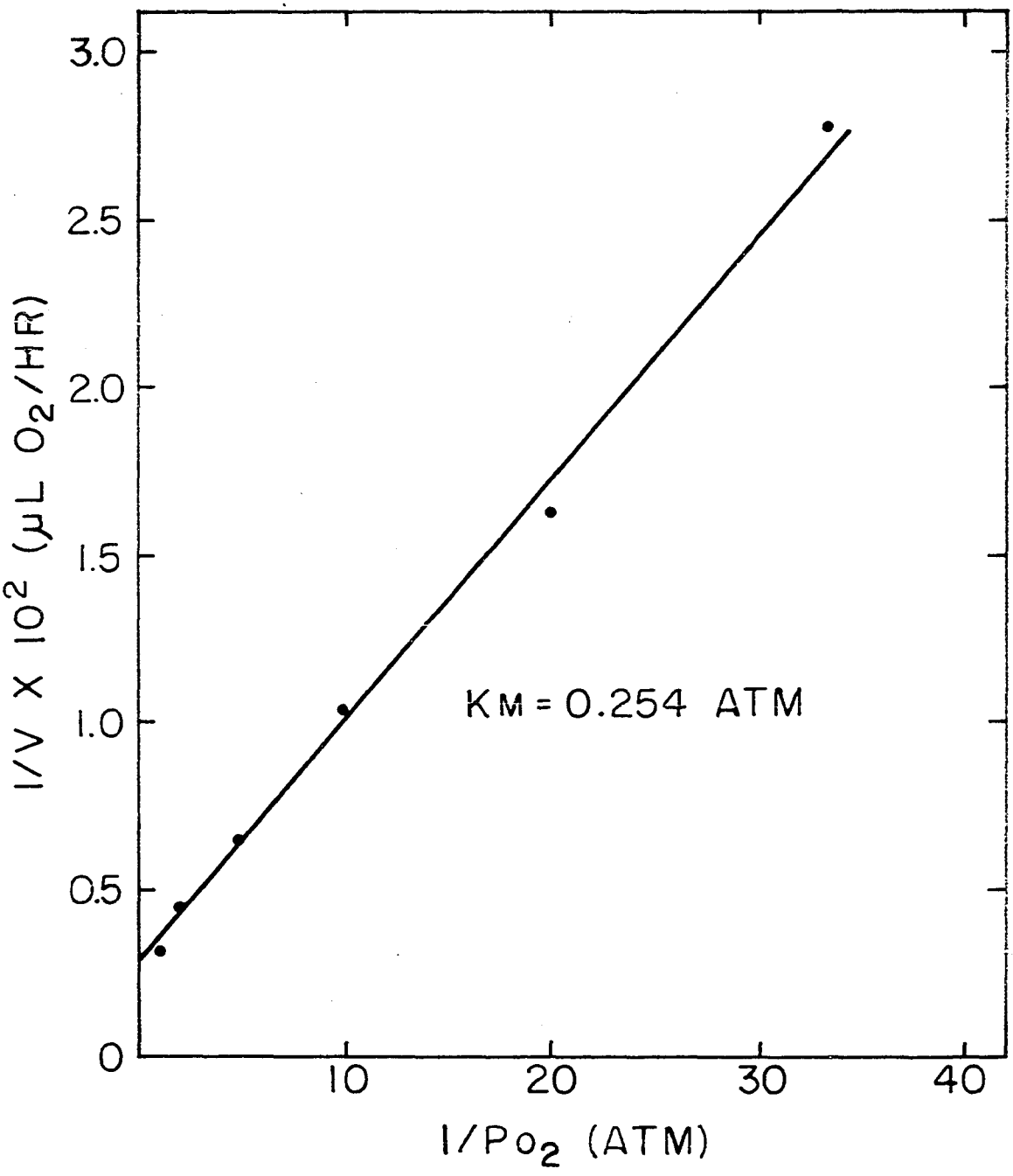
$$O_2^{50} = \frac{pO_2^{50} \times \alpha O_2}{22.4 \text{ litre atm./mole}}$$

where, O_2^{50}: moles O_2/litre needed for half-maximal rate.
αO_2 : Oxygen solubility coefficient (Bunsen coefficient).

[d]Mycelium in moist gas phase.

[e]Mycelium suspended in buffer.

Figure 16. Lineweaver-Burk reciprocal plot of the enzyme extract curve in Figure 15. K_m is the pressure of O_2 in atmospheres needed for half-maximal velocity.


3.0
2.5
2.0
1.5
1.0
0.5
0
1/V X 10² (μL O₂/HR)
10
20
30
40
1/Po₂ (ATM)
KM = 0.254 ATM

reported a phenol oxidase which had the lowest K_m yet found for a non-cytochrome enzyme.

Darby and Goddard (14) found that the rate of O_2 diffusion through a liquid phase limited the respiration rate of mycelial pellets of M. verrucaria. In their experiments, maximum respiration and terminal oxidase saturation was found at 0.4 atmospheres of O_2. Although Darby and Goddard did not calculate an O_2 affinity value, a Lineweaver-Burk (47) plot of their data gave a pO_2^{50} of 0.1 atmospheres. Other workers (40,79,84) have similarly found a marked dependence on O_2 tension of tissues respiring in buffer in which the pO_2^{50} values were larger than expected for systems utilizing cytochromes. When a moist gas phase was used instead, the rate of respiration of the tissue depended upon the pO_2 only at low pressures, and the observed O_2 affinities were greatly increased. For example, Yocum and Hackett (84) found that the removal of the liquid phase from the respiring Aroid spadix tissue decreased the O_2 pressure required for half-saturation about 100 fold. These differences in tissue O_2 affinity can be explained by the fact that the diffusion coefficient of O_2 in water is 3×10^{-5} times the value in air at 20° C.

Consequently, a liquid phase was avoided in the mycelial respiration experiments reported in this thesis by using thin mats of surface-dried mycelium exposed to a moist gas

phase at 30° C. The relation between mycelial respiration and O_2 tension under these conditions is given in Figure 15. An analysis of variance with 4 different cultures as replicates showed no significant difference in respiration rate between air and 0.01 atmospheres of O_2. That is, maximal respiration and terminal oxidase saturation were supported by 0.01 atmospheres of O_2 in the moist gas phase. Compared to Darby and Goddard's (14) results for pellet respiration in buffer at 0.01 atmospheres of O_2, the corresponding rate in the gas phase was approximately 28 times higher. It was noted that the O_2 tension needed for maximal respiration was not constant among experiments but varied with the mycelial mat thickness and wetness, which apparently affected the rate of O_2 diffusion into the tissue. This dependence of tissue respiration on the diffusion path has also been found for respiring potato slices by Thimann et al. (79). The pO_2^{50} for mycelial respiration under these conditions was lower than 12 micromolar, but no definite value or range of values can be given since there were no data points below 0.01 atmospheres of O_2. However, in several experiments at low O_2 tensions in which the mycelium used up all the available O_2 in the flasks, it was calculated from the reaction curves and the amounts of O_2 remaining in the flasks that the rate at 0.005 atmospheres of O_2 was greater than 50 percent of the maximal rate. Thus, the pO_2^{50} would be lower than

0.005 atmospheres of O_2. The maximum value for the affinity of the mycelium for O_2 falls well within the range of published values for known cytochrome-dependent cells and tissues (1-16 micromolar). Table 11 provides some of these affinity values for several intact tissues and cells. The low K_m's for Aerobacter and bakers' yeast were related to the short O_2 diffusion path in the cells and the relatively high K_m for pea internodes was attributed by Eichenberger and Thimann (23) to a limited rate of O_2 diffusion into the tissue. The O_2 affinity which Chance (9) found for kidney mitochondria appears to be the most acceptable minimal value.

The fact that the measured respiratory rate of the mycelium was maximal at 0.01 atmospheres of O_2 whereas the extracted ascorbic acid oxidase was less than 4 percent saturated with O_2 at this pressure would immediately suggest that cytochrome oxidase mediates the terminal oxidation in this tissue. However, these O_2 affinity relationships do not, as such, provide conclusive evidence for a completely cytochrome-dependent respiration since an enzyme of low O_2 affinity could function in an organism like M. verrucaria which normally grows in air.

Another approach to the problem of determining the role of ascorbic acid oxidase in terminal respiration has been the use of inhibitors. However, one of the main

difficulties in assessing the respiratory role of cytochrome oxidase and ascorbic acid oxidase has been the fact that inhibitors of one enzyme *in vivo* also affect the other enzyme. Honda (38) has nicely shown the difficulties encountered when using non-specific inhibitors for determining the participation of enzymes in respiration. Since the atypical oxidase was relatively insensitive to such inhibitors of cytochrome oxidase as cyanide, azide and CO, it appeared that these could be used to selectively inhibit the cytochrome system and quantitatively assess the role of cytochrome oxidase in mycelial respiration. This, unfortunately, did not prove to be the case since Darby and Goddard (15) found that mycelial respiration was insensitive to 95 percent CO and 0.0003M cyanide and was only 83 percent inhibited by 0.001M azide even though a cytochrome oxidase could be extracted which was typically sensitive to low concentrations of these inhibitors.

Since Hilton (36) found that the succinoxidase system in a particle fraction from the mycelium was completely inhibited by 3 micrograms of SN 5949 (2-hydroxy-3(2-methyloctyl)-1, 4-napthoquinone), it was decided to test this inhibitor and the analogous inhibitor antimycin a on mycelial respiration and on the extracted oxidase. SN 5949 and antimycin a both prevent the reduction of cytochrome c by the Slater factor in electron transport between

cytochromes b and c (10,12,13,70), and are quite specific in this effect. It was hoped that these inhibitors would selectively act on the cytochrome system in the mycelium. Both compounds were dissolved in 95 percent ethanol and were kept in the cold no longer than 24 hours when not used immediately. Table 12 gives the results found in these tests. In experiment 1, the mycelial respiration was inhibited approximately 97 percent by 20 micrograms of SN 5949 or 20 micrograms of antimycin a. Concentrations as low as 10 micrograms were less effective but still inhibited the rate of O_2 uptake by 82-84 percent. A slow recovery of respiration from inhibition by these compounds also was observed. In contrast, the extracted ascorbic acid oxidase was not inhibited by 40 micrograms of SN 5949 or 20 micrograms of antimycin a. Concurrent tests on the extracted oxidase were not made in this experiment. A single experiment (number 3) showed that the surface-localized ascorbic acid oxidase had the same response to SN 5949 as the oxidase *in vitro*. On the basis of the results with SN 5949 and antimycin a, it was indicated that little if any of the terminal respiration proceeds via ascorbic acid oxidase in the mycelium.

Another line of attack was to determine the effect on respiration of the oxidase without affecting the cytochrome system. It was found that this could be done by exposing the mycelium to acid for a short time and measuring the

Table 12. Effect of SN 5949 and antimycin a on the respiration of intact mycelium and on ascorbic acid oxidase activity.

Expt.	Inhibitor	Amount (ug.)	Initial O_2 uptake (ul. O_2/hr.) Respiration	Inhib. (%)	Oxidase	Inhib. (%)
					(in vitro)[a]	
1[b]	Control	--	197	---	440	-
	SN 5949	10	36	82	---	-
	SN 5949	16	16	92	---	-
	SN 5949	20	6	97	438	0
	SN 5949	40	---	---	440	0
	Anti. a	10	32	84	439	0
	Anti. a	16	15	92	---	0
	Anti. a	20	7	97	453	0
2[b]	Control	--	111	---	---	-
	SN 5949	20	0	100	---	-
	Anti. a	20	0	100	---	-
					(in vivo)	
3[c]	Control	--	58	---	124	-
	SN5949	10	16	72	123	0

[a]Each flask contained 0.0112M ascorbate, pH 4.5, 0.034M phosphate-0.017M citrate, SN 5949 and Anti. a dissolved in 95% ethanol, fluid volume 2.0 ml. Control had same volume of ethanol.

[b]Each flask contained mycelium suspension, pH 5.0, 0.034M phosphate, 0.0004M Mg, 0.2 ml. KOH in centre well. Inhibitor dissolved in 95% ethanol, fluid volume 2.8 ml. Control had same volume of ethanol.

[c]Each flask contained mycelium suspension, 0.0100M ascorbate, pH 4.5, 0.032M phosphate-0.016M citrate, 0.0004M Mg, 0.2 ml. KOH in centre well, fluid volume 2.8 ml. SN 5949 in 95% ethanol. Control had same volume of ethanol.

effect on ascorbic acid oxidase *in vivo* and on the endogenous respiration. The previously presented data in Table 10, which gives the results of such experiments, shows that even though there was enough of the oxidase to support the observed rate of tissue respiration, a short-term acid exposure eliminated the oxidase yet had no significant effect on respiration. The respiration remaining after the acid treatment as well as the control respiration could be completely inhibited by 20 micrograms of SN 5949. The acid treatment data confirm the results and conclusion using SN 5949 and antimycin a that the terminal oxidase in *M. verrucaria* respiration was not ascorbic acid oxidase but cytochrome oxidase.

Substrate Specificity of the Mycelial, Spore and Cu Enzymes

Enzymes are adapted to catalyze only certain chemical reactions and in this respect are highly specific compared to inorganic catalysts. The essential problem of substrate specificity is to determine the number and nature of the points of interaction between the substrate molecule and the enzyme since these points determine the specificity and allow substrate activation. Since the specific action of an enzyme is determined by definite chemical structures and spatial arrangements of the polar and non-polar groups on the enzyme and substrate, then changing the substrate

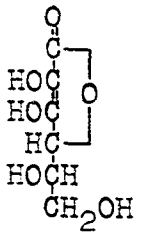
structure and observing the effect of these changes on enzyme action may provide information about the nature of the reactive site. The following work in this thesis describes the substrate specificity of the atypical ascorbic acid oxidase in the spores and mycelium of M. verrucaria and of the typical Cu enzyme from cucumber tissue.

Mandels (52) found that the spore enzyme oxidized L-ascorbate but not D-araboascorbate or D-glucoascorbate while the Cu enzyme from squash oxidized both of these analogs. A test of these compounds on the mycelial enzyme gave the interesting result that L-ascorbate and D-araboascorbate were substrates but not D-glucoascorbate, indicating a specificity difference between the spore and mycelial enzymes. This finding stimulated the collection of a wide variety of rare ascorbate analogs. Since these analogs were available, previous work (20,42,73) on the substrate specificity of unpurified cucumber ascorbic acid oxidase was confirmed and extended to include 7 more analogs. The number of tests on the spore and mycelial enzymes with some of the analogs was limited due to insufficient amounts of these compounds. However, close checks on the condition of the analogs by dye titration and oxidation by the Cu enzyme served to minimize the chances of error.

The structures of the ascorbate analogs tested in the experiments are given as Fischer projections in Figure 17.

Figure 17. Structures of ascorbate analogs.

I	L-Ascorbate (L-xyloascorbate)
II	6-Deoxy-L-ascorbate
III	D-Xyloascorbate
IV	D-Araboascorbate
V	L-Araboascorbate
VI	L-Glucoascorbate
VII	D-Glucoascorbate
VIII	Imino-D-glucoascorbate
IX	L-Rhamnoascorbate
X	L-Galactoascorbate
XI	D-Galactoascorbate
XII	Imino-D-galactoascorbate
XIII	L-Guloascorbate
XIV	Imino-L-guloascorbate
XV	L-Erythroascorbate
XVI	Hydroxytetronate
XVII	Reductate
XVIII	Methyl reductate
XIX	Reductone
XX	Dihydroxyfumarate



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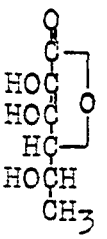

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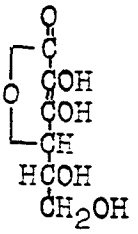

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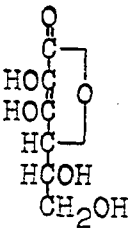

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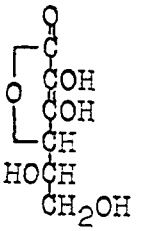

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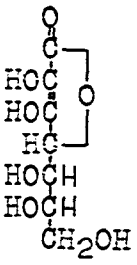

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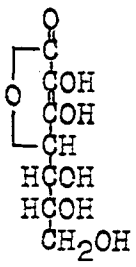

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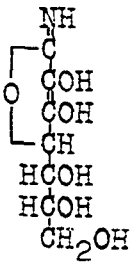

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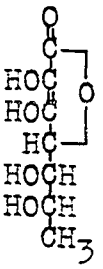

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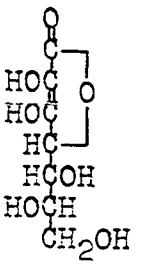

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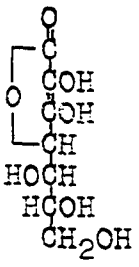

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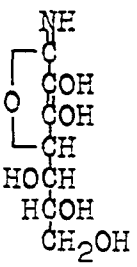

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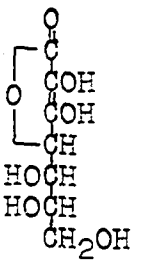

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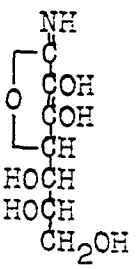

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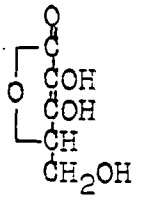

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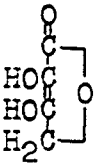

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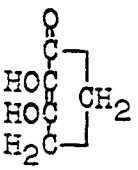

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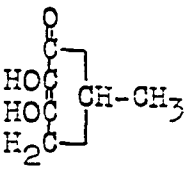

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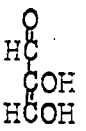


XIX

XX

Since some of the rare analogs were old preparations subject to autoxidation, they were analyzed for the amount of reduced form remaining. The percentage of reduced form was determined either by triplicate dye titration using 2, 6-dichlorophenol indophenol with ascorbate as the standard or by enzymatic estimation using cucumber oxidase which had a stoichiometry of one-half mole of O_2 uptake per mole of analog. Calculations from the dye titrations were made on the assumption that the analogs had the same reducing properties as L-ascorbate, reacting with 1 mole of dye oxidant per mole of analog. Table 13 shows that most of the compounds were over 90 percent reduced and thus were in usable condition despite storage of some for more than 20 years. The analog concentrations in the reaction mixtures were corrected for non-reduced substrate.

Before setting up experiments comparing the initial rates of L-ascorbate versus ascorbate analog oxidation, it was necessary to find the concentrations of analogs required for enzyme saturation or maximal reaction velocity. Figure 18 shows the effect of concentration on initial reaction rates in air of the mycelial enzyme for those substrates available in quantity. The rates were corrected for autoxidation where necessary. Figure 19 is a Lineweaver-Burk (47) reciprocal plot of the data in Figure 18 with the calculated K_m values given in Table 14. The mycelial enzyme had

Table 13. Estimation of the percent reduced form of the ascorbate analogs.

Analog	Method[a]	Micromoles		% Reduced form
		Calc.	Obs.	
L-Ascorbate	titr.	0.171	0.171	100
D-Xyloascorbate	titr.	0.223	0.200	90
D-Araboascorbate	titr.	0.256	0.256	100
L-Araboascorbate	titr.	0.165	0.223	100
D-Glucoascorbate	titr.	0.284	0.262	92
Imino-D-glucoascorbate	titr.	0.398	0.176	44
D-Galactoascorbate	titr.	0.136	0.151	100
Imino-D-galactoascorbate	titr.	0.483	0.409	85
Imino-L-guloascorbate	titr.	0.568	0.085	15
Hydroxytetronate	titr.	0.210	0.204	97
Reductate	titr.	0.142	0.131	92
Methyl reductate	titr.	0.224	0.182	81
Reductone	titr.	0.125	0.114	91
Dihydroxyfumarate	titr.	0.150	0.080	53
6-Deoxy-L-ascorbate	enzyme	5.00	5.36	100
L-Glucoascorbate	enzyme	4.82	5.00	100
L-Rhamnoascorbate	enzyme	11.05	8.94	80
L-Galactoascorbate	enzyme	4.30	5.53	100
L-Guloascorbate	enzyme	4.82	4.37	91
L-Erythroascorbate	enzyme	6.42	5.98	93

[a]Titration using 2, 6-dichlorophenol indophenol dye. Enzyme analysis using cucumber ascorbic acid oxidase.

approximately the same affinity for L-ascorbate, D-araboascorbate, hydroxytetronate and reductate but a lower affinity for methyl reductate and reductone. Sufficient amounts of the other analogs were not available to set up similar saturation curves; but assuming that the enzyme affinity for these analogs and L-ascorbate was approximately the same, the concentrations of the active substrates tested gave maximal velocity. These concentrations ranged from

Figure 18. Effect of substrate concentration on initial reaction rate of the mycelial enzyme.

Curve	Substrate
1	L-Ascorbate
2	Hydroxytetronate
3	Reductate
4	D-Araboascorbate
5	Methyl reductate
6	Reductone

Reaction conditions: pH 4.5, 0.034M phosphate-0.017M citrate.

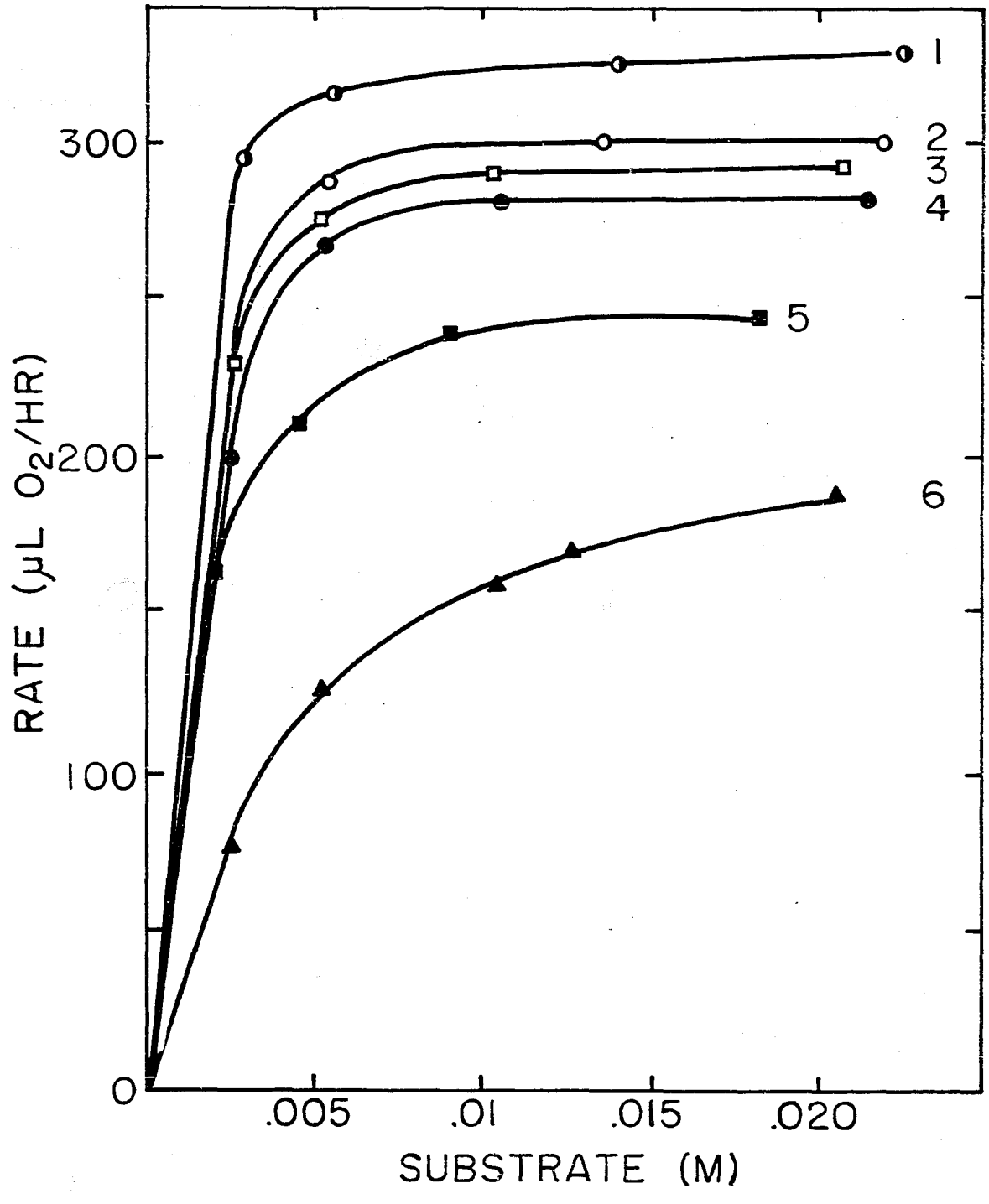

RATE (μL O2/HR)
300
200
100
0
.005
.01
.015
.020
SUBSTRATE (M)
1
2
3
4
5
6

Figure 19. Lineweaver-Burk reciprocal plots of reaction rate as a function of analog concentration for the data in Figure 18.

Curve	Symbol	Substrate
1	half-closed circles	L-Ascorbate
2	open circles	Hydroxytetronate
2	open squares	Reductate
3	closed circles	D-Araboascorbate
4	closed squares	Methyl reductate
5	closed triangles	Reductone

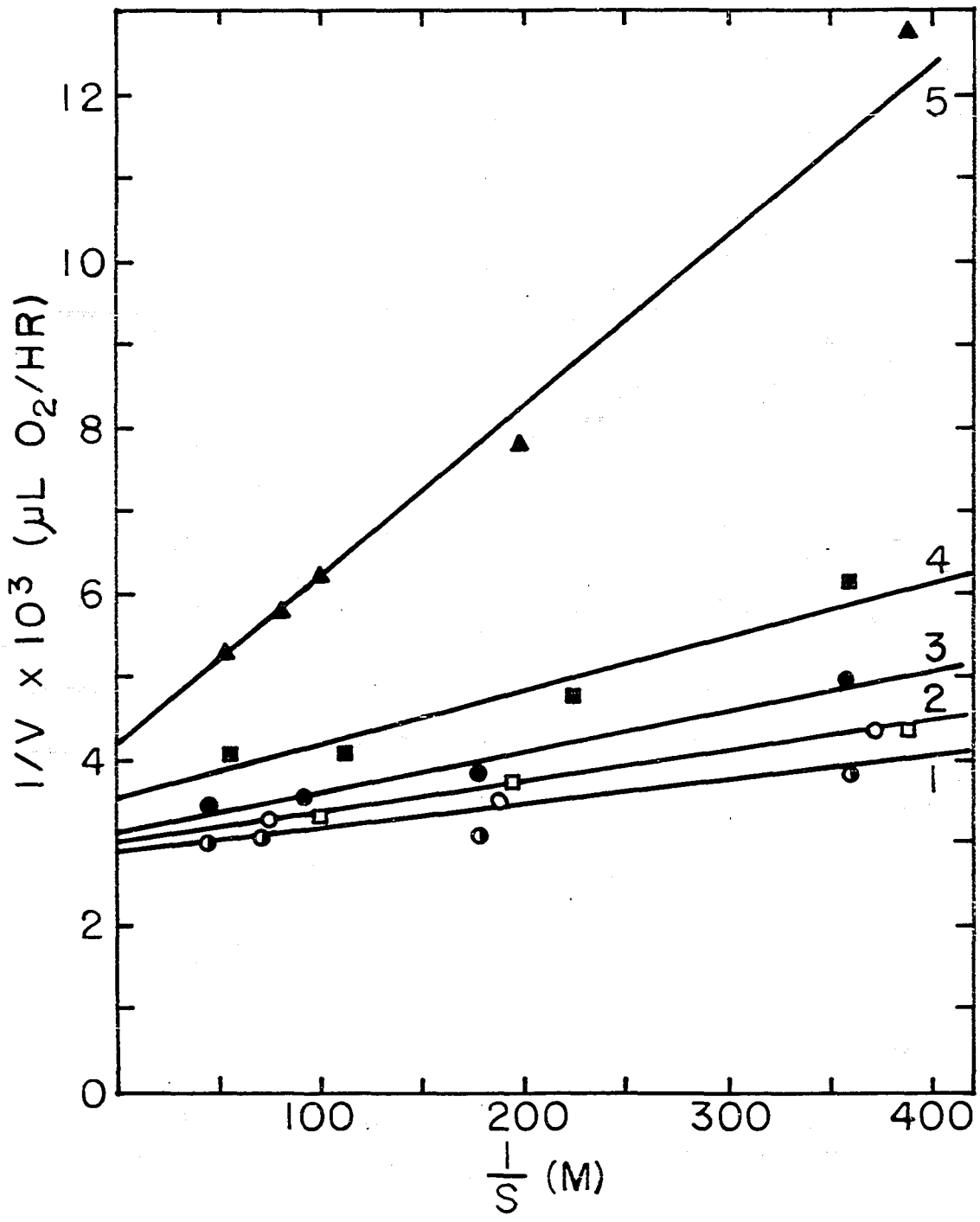

1/V x 10³ (μL O₂/HR)
0
2
4
6
8
10
12
100
200
300
400
1/S (M)
5
4
3
2
1

Table 14. K_m values for the mycelial enzyme and several ascorbate analogs. (Calculated from the reciprocal plots in Figure 19.)

Substrate	K_m
L-Ascorbate	0.0013M
Hydroxytetronate	0.0013M
Reductate	0.0013M
D-Araboascorbate	0.0016M
Methyl reductate	0.0018M
Reductone	0.0052M

0.0091M to 0.0207M. Mandels (52) reported that the spore enzyme was saturated at about 0.004M ascorbate while Snow and Zilva (73) found that the Cu enzyme was saturated at about 0.01M with L-ascorbate and various ascorbate analogs. On the basis of these results, the spore and Cu oxidases tested in the following experiments were adequately saturated.

Spore enzyme

Mandels (52) made the cautious suggestion from his limited data on substrate specificity that the spore enzyme might be absolutely specific for L-ascorbate. This suggestion has turned out to be right so far. The evidence is shown in Table 15 which gives the relative activities of different ascorbate analogs as substrates for the spore enzyme. Each experiment represents a different enzyme preparation. All the reactions were allowed to run at least 60 minutes to determine if there was an initial lag period

Table 15. Relative activity of ascorbate analogs as substrates for the spore enzyme.

Expt.	Substrate	Conc.[a] (M)	Rate[b] (ul.O_2/hr.)	Activity[c] (%)
1	L-Ascorbate	0.0112	294	100
	D-Araboascorbate	0.0100	0	0
	L-Araboascorbate	0.0120	0	0
	D-Xyloascorbate	0.0112	0	0
	D-Glucoascorbate	0.0089	0	0
	D-Galactoascorbate	0.0081	0	0
	Dihydroxyfumarate	0.0070	0	0
2[d]	L-Ascorbate	0.0112	60	100
	6-Deoxy-L-ascorbate	0.0098	0,0	0
	L-Erythroascorbate	0.0118	0,0	0
	L-Guloascorbate	0.0088	0,0	0
3[d]	L-Ascorbate	0.0141	80	100
	L-Galactoascorbate	0.0083	0,0	0
	L-Glucoascorbate	0.0096	0,0	0
	L-Rhamnoascorbate	0.0174	0,0	0
4[d]	L-Ascorbate	0.0141	270	100
	Imino-D-glucoascorbate	0.0062	0	0
	Imino-D-galactoascorbate	0.0041	0	0
	Imino-L-guloascorbate	0.0021	0	0

[a]Corrected for non-reduced substrate. Each flask contained pH 6.0, 0.034M phosphate-0.017M citrate.

[b]Initial rate.

[c]Percent of the rate of L-ascorbate oxidation.

[d]Measurements made in 5.0 ml. flasks containing a fluid volume of 0.5 ml. Duplicate rate values were from individual flasks.

Table 15. (Continued)

Expt.	Substrate	Conc. (M)	Rate ($ul.O_2/hr.$)	Activity (%)
5	L-Ascorbate	0.0141	312	100
	Hydroxytetronate	0.0137	0	0
	Reductate	0.0130	0	0
	Methyl reductate	0.0114	0	0
	Reductone	0.0128	0	0
6	L-Ascorbate	0.0226	256	100
	Hydroxytetronate	0.0220	0	0
	Reductate	0.0208	0	0
	Methyl reductate	0.0183	0	0
	Reductone	0.0206	0	0
7	L-Ascorbate	0.0112	235	100
	Hydroxytetronate	0.0125	0	0
	Methyl reductate	0.0091	0	0
8	L-Ascorbate	0.0112	228	100
	Reductate	0.0103	0	0
	Reductone	0.0102	0	0

in oxidation.

Mandels' results (52) which showed no oxidation of either D-araboascorbate or D-glucoascorbate were confirmed in experiment 1. From the impressive list of zero oxidation rates in this and the following experiments, it can be seen that the spore enzyme oxidized only L-ascorbate among the 20 structural analogs tested. In agreement with Mandels' data, the stoichiometry for the spore enzyme was one-half mole of O_2 uptake per mole of analog. In experiment 1, the spore enzyme also failed to oxidize any of the 6- or 7-carbon analogs having the opposite C-4 configuration to L-ascorbate, L-araboascorbate (V), D-xyloascorbate (III), D-glucoascorbate (VII) and D-galactoascorbate (XI). The trans-dienol dihydroxyfumarate (XX) was inactive as a substrate. In experiment 2, 7-carbon L-guloascorbate (XIII) and 5-carbon L-erythroascorbate (XV), which also had the opposite C-4 configuration to L-ascorbate, were not oxidized. Experiment 4 showed that the imino derivatives of 3 of these 7-carbon analogs were inactive. Substituting the hydroxyl group at C-6 on L-ascorbate with a hydrogen to give 6-deoxy-L-ascorbate (II) likewise produced an inactive substrate. Of conclusive importance in the elucidation of the spore enzyme specificity was the fact that none of the 7-carbon analogs with the same C-4 configuration as L-ascorbate were oxidized, regardless of the C-5 hydroxyl position and

the C-7 substituent group. Experiment 3 gives such results when L-galactoascorbate (X), L-glucoascorbate (VI) and L-rhamnoascorbate (IX) were tried. Experiments 5-8 showed that the enzyme would not oxidize the 4-carbon analog, hydroxytetronate (XVI), which lacks the C-5 hydroxyl and the C-6 primary alcohol groups of L-ascorbate. Replacing the ring oxygen with a methylene group or a methyl-substituted methylene group to give reductate (XVII) and methyl reductate (XVIII), respectively, or removing the ring entirely to give reductone (XIX) did not produce an active structure.

From these results it appears that the only compound the spore enzyme can oxidize is L-ascorbate. The principle question now remaining is whether the carbonyl group is required for activity. The high degree of specificity implies a very exact attachment of the L-ascorbate molecule to the active site on the enzyme.

Mycelial enzyme

Testing the same analogs on the mycelial enzyme gave the results listed in Table 16, each experiment representing a different enzyme preparation. Experiments 1-8 showed that the mycelial enzyme did not oxidize any of the 6- and 7-carbon structures having the opposite C-4 configuration to L-ascorbate, L-araboascorbate (V), D-xyloascorbate (III), D-glucoascorbate (VII), D-galactoascorbate (XI) and L-guloascorbate (XIII). In addition to these analogs in

Table 16. Relative activity of ascorbate analogs as substrates for the mycelial enzyme.

Expt.	Substrate	Conc.[a] (M)	Rate[b] ($\mu l. O_2/hr.$)	Activity[c] (%)
1	L-Ascorbate	0.0112	270	100
	D-Araboascorbate	0.0100	168	62
	L-Araboascorbate	0.0112	0	0
	D-Xyloascorbate	0.0100	0	0
	D-Glucoascorbate	0.0088	0	0
	D-Galactoascorbate	0.0096	0	0
	Dihydroxyfumarate	0.0070	0	0
2	L-Ascorbate	0.0112	295	100
	D-Araboascorbate	0.0100	260	88
	L-Araboascorbate	0.0112	4	1
	D-Glucoascorbate	0.0088	0	0
	D-Galactoascorbate	0.0096	6	2
	Dihydroxyfumarate	0.0070	0	0
3	L-Ascorbate	0.0112	62	100
	D-Xyloascorbate	0.0202	0	0
4	L-Ascorbate	0.0112	420	100
	L-Araboascorbate	0.0084	0	0
	D-Glucoascorbate	0.0088	0	0
	Imino-D-glucoascorbate	0.0042	0	0
	D-Galactoascorbate	0.0072	10	2
5[d]	L-Ascorbate	0.0112	126	100
	6-Deoxy-L-ascorbate	0.0098	60,66	50
	L-Erythroascorbate	0.0118	18,28	23
	L-Guloascorbate	0.0088	0	0

[a]Corrected for non-reduced substrate. Each flask contained pH 4.5, 0.034M phosphate-0.017M citrate.

[b]Initial rate.

[c]Percent of rate of L-ascorbate oxidation.

[d]Measurements made in 5.0 ml. flasks containing a fluid volume of 0.5 ml. Duplicate rate values were from individual flasks.

Table 16. (Continued)

Expt.	Substrate	Conc. (M)	Rate (ul.O_2/hr.)	Activity (%)
6[d]	L-Ascorbate	0.0112	105	100
	L-Araboascorbate	0.0112	0	0
	L-Guloascorbate	0.0088	0	0
	D-Galactoascorbate	0.0097	0	0
	Imino-D-galactoascorbate	0.0041	0	0
7[d]	L-Ascorbate	0.0112	80	100
	L-Rhamnoascorbate	0.0174	72,78	98
	L-Glucoascorbate	0.0100	72	90
	L-Galactoascorbate	0.0083	66,94	100
8	L-Ascorbate	0.0141	314	100
	Imino-D-glucoascorbate	0.0062	0	0
	Imino-L-guloascorbate	0.0021	0	0
9	L-Ascorbate	0.0141	332	100
	Hydroxytetronate	0.0125	280	84
	Reductate	0.0130	291	88
	Methyl reductate	0.0114	250	75
	Reductone	0.0128	172	52
10	L-Ascorbate	0.0141	346	100
	Hydroxytetronate	0.0137	300	87
	Reductate	0.0130	290	84
	Methyl reductate	0.0114	280	81
11	L-Ascorbate	0.0112	316	100
	Hydroxytetronate	0.0125	300	95
	Reductate	0.0162	290	92
	Methyl reductate	0.0126	250	79
	Reductone	0.0207	200	63
12	L-Ascorbate	0.0112	316	100
	Reductate	0.0104	280	89
	Methyl reductate	0.0091	240	76
13	L-Ascorbate	0.0141	334	100
	Hydroxytetronate	0.0137	292	87
	Reductate	0.0130	242	72
	Methyl reductate	0.0114	240	72
	Reductone	0.0128	180	54

experiments 1-8, dihydroxyfumarate, imino-D-glucoascorbate, imino-D-galactoascorbate and imino-L-guloascorbate were inactive. As seen in experiment 7, the 7-carbon analogs with the same ring configuration as L-ascorbate, L-glucoascorbate (VI), L-rhamnoascorbate (IX) and L-galactoascorbate (X), were oxidized approximately as fast as L-ascorbate. D-araboascorbate (IV), which has the opposite C-5 hydroxyl configuration to L-ascorbate, gave activity values ranging from 38-88 percent in 8 experiments. This was the only analog which was oxidized at such widely varying rates by different enzyme preparations. Experiment 5 with 6-deoxy-L-ascorbate (II) showed that the substitution of a hydrogen for the hydroxyl group at C-6 on L-ascorbate decreased the relative rate by 50 percent. L-Erythroascorbate (XV) also was found to be slowly oxidized (23 percent) in experiment 5. This was particularly interesting since L-erythroascorbate had the opposite C-4 configuration to L-ascorbate and was the only analog with this configuration which was active. Removing the side chain or replacing the ring oxygen with a methylene group had little effect on activity since hydroxytetronate (XVI) and reductate (XVII) were oxidized about 85 percent as rapidly as L-ascorbate. Methyl reductate (XVIII) was almost as active. The simplest dienol oxidized was reductone (XIX) which had a dienol group adjacent to a carbonyl group. Experiments 9-13 give the results obtained

with these last 4 analogs. The stoichiometry of analog oxidation was one-half mole of O_2 uptake per mole of analog, indicating that oxidation of the analogs beyond the dehydro stage did not occur. Rate curves for the mycelial oxidase and several representative ascorbate analogs are given in Figure 20. In each case, the use of initial rates appeared to be a valid basis for comparing relative activities. The general shape of the reaction curves was similar for every analog attacked except reductone. As seen in Figure 20, the rate of reductone oxidation fell off completely even before stoichiometry was reached. At the present time no explanation can be given for this result nor the finding that reductone at pH 4.5 had an unusually high autoxidation rate of 70-100 microliters of O_2 per hour.

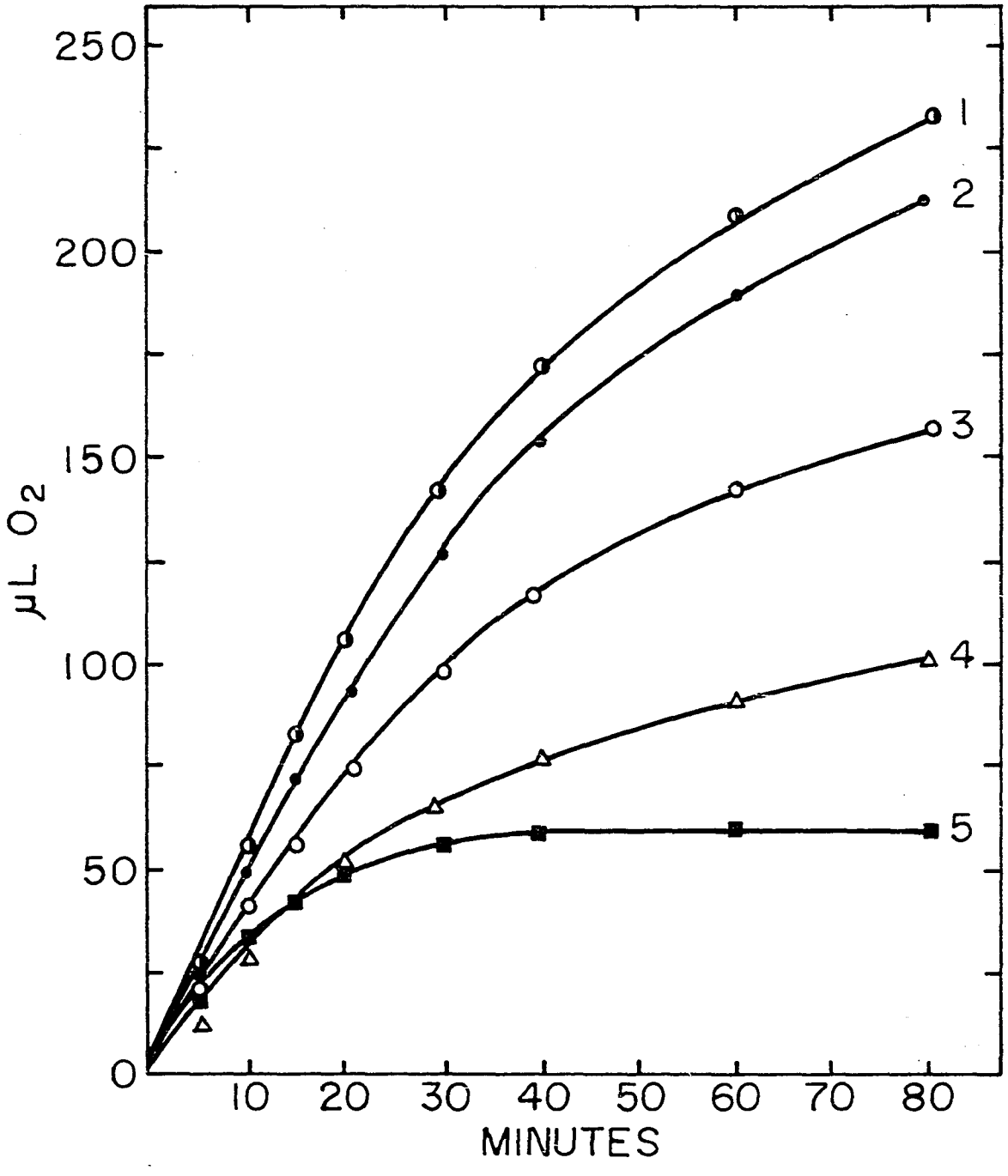
The mycelial enzyme was far less specific for the L-ascorbate structure than the spore enzyme. Reductone had the minimum structural requirements for activity. These were the dienol group and possibly the carbonyl oxygen atom although the latter requirement can not be determined until an appropriate C-1 substituted analog is tested. The side chain obviously had some effect on activity since hydroxytetronate (XVI) was oxidized more slowly than L-ascorbate. When the side chain is present, the structure essential for full activity appears to be a molecule with the same C-4 configuration as L-ascorbate and at least 1 hydroxyl group

Figure 20. Rates curves for the mycelial enzyme and various ascorbate analogs and for the spore enzyme and ascorbate.

Curve	Enzyme	Substrate	(M)
1	Mycelial	L-Ascorbate	0.0140
2	Mycelial	Reductate	0.0140
3	Spore	L-Ascorbate	0.0112
4	Mycelial	D-Araboascorbate	0.0100
5	Mycelial	Reductone	0.0140

Reaction conditions for mycelial enzyme: pH 4.5, 0.034M phosphate-0.017M citrate.

Reaction conditions for spore enzyme: pH 6.0, 0.034M phosphate-0.017M citrate.


250
200
150
100
50
0
µL O2
10
20
30
40
50
60
70
80
MINUTES
1
2
3
4
5

which is adjacent to a primary or secondary alcohol group and to the left of the side chain in a Fischer projection. However, this can not be definitely established until an analog such as D-alloascorbate with both hydroxyl groups on C-5 and C-6 to the right of the chain is tested.

Cu enzyme

Johnson and Zilva (42) and Snow and Zilva (73) found that unpurified ascorbic acid oxidase from cucumber tissue would oxidize ascorbate analogs at rates dependent on the configuration of the lactone ring and the length of the carbon chain. Essentially the same results have been found by other workers (19,20) using partially purified or purified ascorbic acid oxidases from higher plants. The experiments with crude cucumber juice summarized in Table 17 are in close agreement with these early findings. Each experiment represents a different enzyme preparation. The rates were generally constant until the substrate was depleted except for some analogs where they gradually fell off with time. Therefore, initial velocities were used in the calculation of relative activities.

The number of analogs previously tested on the cucumber enzyme was extended to include 6-deoxy-L-ascorbate, L-rhamnoascorbate, L-guloascorbate, imino-D-glucoascorbate, imino-D-galactoascorbate, imino-L-guloascorbate, hydroxytetronate and methyl reductate. Experiments 1-5 in Table 17

Table 17. Relative activity of ascorbate analogs as substrates for the Cu enzyme.

Expt.	Substrate	Conc.[a] (M)	Rate[b] (ul.O_2/hr.)	Activity[c] (%)
1	L-Ascorbate	0.0112	330	100
	D-Araboascorbate	0.0100	330	100
	L-Araboascorbate	0.0120	112	34
	D-Xyloascorbate	0.0111	214	65
	D-Glucoascorbate	0.0089	62	19
	D-Galactoascorbate	0.0081	90	27
	Dihydroxyfumarate	0.0070	0	0
2[d]	L-Ascorbate	0.0141	288	100
	L-Rhamnoascorbate	0.0174	264,291	97
	L-Galactoascorbate	0.0083	276,282	97
3[d]	L-Ascorbate	0.0141	258	100
	L-Glucoascorbate	0.0096	234	91
4[d]	L-Ascorbate	0.0112	276	100
	6-Deoxy-L-ascorbate	0.0098	312	113
	L-Erythroascorbate	0.0118	126	46
	L-Guloascorbate	0.0088	96	35

[a]Corrected for non-reduced substrate. Each flask contained pH 6.0, 0.034M phosphate-0.017M citrate.

[b]Initial rate.

[c]Percent of rate of L-ascorbate oxidation.

[d]Measurements made in 5.0 ml. flasks containing a fluid volume of 0.5 ml. Duplicate rate values were from individual flasks.

Table 17. (Continued)

Expt.	Substrate	Conc. (M)	Rate ($ul.O_2/hr.$)	Activity (%)
5	L-Ascorbate	0.0141	618	100
	Imino-L-guloascorbate	0.0021	0	0
	Imino-D-glucoascorbate	0.0062	0	0
	Imino-D-galactoascorbate	0.0041	0	0
6	L-Ascorbate	0.0141	432	100
	Hydroxytetronate	0.0137	414	96
	Reductate	0.0130	276	64
	Methyl reductate	0.0114	192	45
	Reductone	0.0128	272	63
7	L-Ascorbate	0.0141	450	100
	D-Araboascorbate	0.0141	504	112
	Reductate	0.0131	288	64
	Methyl reductate	0.0114	234	62
	Reductone	0.0128	201	45
8	L-Ascorbate	0.0141	374	100
	Reductate	0.0130	210	56
	Methyl reductate	0.0114	164	44
	Reductone	0.0128	188	50
9	L-Ascorbate	0.0141	418	100
	Hydroxytetronate	0.0137	460	110

give the initial rates of oxidation of the 6- and 7-carbon analogs. Analogs with the same ring configuration as L-ascorbate, 6-deoxy-L-ascorbate (II), D-araboascorbate (IV), L-glucoascorbate (VI), L-rhamnoascorbate (IX) and L-galactoascorbate (X), were oxidized at rates as fast as L-ascorbate and linearly with time. The chain length, hydroxyl configuration at C-5 or C-6 or the lack of a primary alcohol group at C-6 or C-7 had no influence on the activity. The 6-carbon analogs with the opposite ring configuration to L-ascorbate, D-xyloascorbate (III) and L-araboascorbate (IV), were oxidized 2-3 times faster than the 7-carbon members of the same series, D-glucoascorbate (VII) and D-galactoascorbate (XI), but more slowly than L-ascorbate. The oxidation rates of these 6- and 7-carbon analogs gradually fell off from zero time. The amounts of L-erythroascorbate and L-guloascorbate tested did not permit a valid estimation of fall off in oxidation rate. Dihydroxyfumarate (XX) was inactive presumably due to the trans-dienol structure. None of the imino ascorbate analogs were oxidized adding support to Dodd's (20) conclusion that a free C-1 carbonyl group is required for enzyme-substrate binding and activation. Experiments 6-9 showed that the cucumber enzyme oxidized hydroxytetronate as rapidly as L-ascorbate but oxidized reductate, methyl reductate and reductone at lower rates. Hydroxytetronate was oxidized

at a constant rate while the rates of O_2 uptake for the other 3 analogs fell off gradually from zero time.

The Cu enzyme was less specific than the mycelial enzyme, the main difference being the lack of specificity for a particular C-4 configuration. The only apparent structural requirements for full activity were a free C-1 carbonyl group and a cis-dienol group. Hydroxytetronate (XVI) had the minimum molecular structure needed for full activity.

A concise summary and average of the activity values in Tables 15, 16 and 17 is given in Table 18. In brief, the outcome of the specificity studies was to provide almost conclusive evidence that the spore enzyme had an absolute specificity for L-ascorbate while the mycelial and Cu enzymes were less specific in that they oxidized a wider range of ascorbate analogs. Some speculation and ideas on the stereochemistry of enzyme-substrate attachment and the points of interaction are presented in the Discussion.

Mandels (52) reported that the spore enzyme was irreversibly inhibited by D-araboascorbate but not by D-glucoascorbate. This result was intriguing from the viewpoint that a study of inhibition of enzyme reactions with molecules of structure similar to the active substrate may provide some information about the structure of the reaction site and the nature of the activated complex. In single experiments on this thesis, it was found that the only other

Table 18. Summary of the relative activities of ascorbic acid oxidases. (Activity values averaged from the data in Tables 15, 16 and 17.)

Substrate	Relative enzyme activity (%)		
	Spore	Mycelium	Cucumber
L-Ascorbate	100	100	100
6-Deoxy-L-ascorbate	0	50	113
D-Xyloascorbate	0	0	65
D-Araboascorbate	0	38-88	107
L-Araboascorbate	0	0	34
L-Glucoascorbate	0	90	91
D-Glucoascorbate	0	0	19
Imino-D-glucoascorbate	0	0	0
L-Rhamnoascorbate	0	98	97
L-Galactoascorbate	0	100	97
D-Galactoascorbate	0	0	27
Imino-D-galactoascorbate	0	0	0
L-Guloascorbate	0	0	35
Imino-L-guloascorbate	0	0	0
L-Erythroascorbate	0	23	46
Hydroxytetronate	0	88	104
Reductate	0	84	62
Methyl reductate	0	77	46
Reductone	0	55	52
Dihydroxyfumarate	0	0	0

ascorbate analogs which appeared to inhibit the spore enzyme were 6-deoxy-L-ascorbate and L-galactoascorbate. Single tests of the analogs on the mycelial enzyme showed no inhibition of L-ascorbate oxidation. However, these results are only tentative and more investigation is needed.

DISCUSSION

The main object of this study was to determine the general properties of the mycelial ascorbic acid oxidase and to ascertain the role of the enzyme in mycelial respiration. In addition, certain properties of the enzyme were compared with those of the spore and Cu oxidases. The enzyme was easily obtained in solution by grinding the mycelium with glass and buffer in a Potter-Elvehjem homogenizer. It was then necessary to centrifuge this crude extract at 20,000 x g. to remove particles containing cytochrome oxidase. Since neither cytochrome oxidase or polyphenol oxidase could be detected in the extract following high speed centrifugation, the added ascorbate was directly oxidized by ascorbic acid oxidase. The enzymatic nature of the oxidation of ascorbate by the mycelial extract was demonstrated by heat lability and the failure of dialysis to remove activity. The latter also showed that no soluble intermediate was involved in electron transfer as postulated by Ward (81) for the atypical oxidase in Physarum. The stoichiometry of the reaction also showed that the oxidation of ascorbate was not due to non-enzymatic, heavy-metal catalysis (74).

One interesting result was that the mycelial oxidase separated into a soluble and an insoluble fraction at pH 4.5. The insoluble enzyme was not merely carried down by other protein since extensive washings with buffer solution

failed significantly to diminish the activity of the fraction. It would appear that the insoluble fraction represents a partially denatured form of the enzyme. No constant proportion of soluble to insoluble fraction was found, and the only difference observed between the two fractions was in response to pH and several inhibitors. Purification of the enzyme in both fractions is obviously needed before any conclusions about their nature can be reached.

The pH-optimum of the mycelial enzyme in phosphate-citrate buffer is lower and has a narrower range than is reported for the Cu oxidase and the spore enzyme. Both the Cu and spore enzyme have a pH optimum of 5.6 in phosphate-citrate buffer versus 4.5 for the mycelial enzyme. Since ascorbate has two ionizable acid groups, it might be expected that kinetic effects due to the changing degree of ionization would be observed. That is, the enzyme might attack only the undissociated molecule or one of the dissociated ions. The interpretation of such effects is complicated by the possibility that the enzymatic site may ionize in the pH range under investigation. From the nature of the pH curves, ionization of the enzyme appears to be involved, and it is assumed that the steep portion of the pH-dependence curve corresponds to the dissociation of ionizing groups on the enzyme. The pH data suggest that the monovalent ascorbate ion is attacked since the enzyme is still approximately

50 percent active at pH 5.6 where the monovalent ascorbate ion constitutes 97 percent of the total ascorbic acid. Lu Valle and Goddard (49) also have suggested that Cu oxidase catalysis involves the monovalent ascorbate ion, however, no detailed analysis has been made to confirm this.

The Michaelis constant of the mycelial enzyme in air was approximately 30 times greater than the one found by Frieden and Maggiolo (29) for the Cu oxidase. The fact that the K_m of the mycelial enzyme increased with O_2 tension showed that it does not represent a true equilibrium constant of the enzyme-substrate reaction. This simply means that the equilibrium is shifted toward formation of the enzyme-substrate complex whose decomposition depends on the acceptor concentration. This situation appears analogous to the one Chance (8) found for catalase and peroxidase. The rate of ascorbate oxidation increased with O_2 tension as was the case with the Cu and spore enzymes (23,29,52,79). The concentration of O_2 in the liquid phase proved to be the rate-limiting factor, the rate in air being approximately 40-50 percent of the rate in O_2. The rate of reaction at 1 atmosphere O_2 was only about 90 percent of V_{max}. Such effects of O_2 tension were explained when the O_2 affinity (K_m) of the enzyme was determined to be 0.0003M, while the molar concentration of O_2 in the liquid phase at 30° C was only 0.00024. Thus the properties of the enzyme were not

determined under reaction conditions which allowed maximal velocity. This is the usual experimental case with oxidases having low O_2 affinities.

In the identification and characterization of an enzyme, use is often made of certain inhibitors which are known to act on specific types of enzymes as, for example, sulfhydryl or metal-containing enzymes. The results of such tests often indicate the nature of the proshtetic group of the enzyme or provide information about the nature of essential groups on the protein. As a rule, oxidases involved in the direct transfer of electrons to molecular O_2 have some metal functioning in the process. Thus it was logical to postulate that the mycelial enzyme had a metal prosthetic group. Consequently, a number of metal-enzyme inhibitors were tested on the mycelial oxidase to obtain evidence for such a group. The enzyme proved to be relatively resistant to the common inhibitors of Cu and Fe enzymes and was actually stimulated by several Cu inhibitors. The inhibitor results also showed that the enzyme did not have essential sulfhydryl groups and that polyphenol oxidase and cytochrome oxidase were not oxidizing the added ascorbate. The effects of metal inhibitors, then, clearly differentiated the atypical ascorbic acid oxidase from the Cu enzyme. However, the question of whether a metal ion is involved in electron transfer awaits purification of the enzyme since conclusive evidence in this

respect can not be obtained by working with a crude enzyme extract. The possibility of the mycelial enzyme being a flavoprotein is still open even though there was no evidence of a flavin prosthetic group on the basis of tests with flavin inhibitors. The purity of the enzyme may again be a factor in this case.

The spore enzyme was similar to the mycelial enzyme in response to inhibitors in that it was resistant to various metal-enzyme reagents. Mandels' (51) inhibitor results also suggested the absence of a heavy metal prosthetic group on the spore enzyme.

A kinetic analysis of the spore and mycelial enzyme reactions using the conventional first order equation gave a family of curves which increased in slope with decreasing initial substrate concentration. Unlike a true first order reaction, the reaction "constant" was dependent on the substrate concentration and increased with time. This deviation from first order rate law was more apparent at the higher substrate concentrations where the rate of oxidation approximated zero order kinetics. A decrease in the slope of the curves at the higher substrate levels indicated second order effects apparently due to enzyme inactivation. The integrated form of the Michaelis-Menton equation was used to describe the kinetic situation. This equation combines the first and zero order effects to give an integrated

reaction constant k' which, as Elkins-Kaufmann and Neurath (24) point out, should be independent of the enzyme and substrate concentration and the reaction time. However, the k'e value of the mycelial enzyme was dependent on the concentration of both ascorbate and O_2 and varied with reaction time. The effect of ascorbate concentration on initial k'e values for the mycelial enzyme was more obvious at a high O_2 tension (1 atm.) which allowed near-maximal velocities. Under these conditions the rate of enzyme inactivation was dependent on the reaction velocity as reflected by the change in the initial k'e values. The value of the mixed-order plot in these experiments is that it takes out the effect of substrate concentration on reaction order and by doing so allows an analysis of the change in k'e due to enzyme inactivation. More experiments are needed at this stage to analyze kinetically enzyme inactivation as a function of the ascorbate and O_2 concentration. Considering the nature of the system, the reaction constant k' and the turnover number of the enzyme would be expected to increase with O_2 concentration, providing the ascorbate concentration is not rate-limiting.

The semilog plot of the k'e values versus time showed that the rate of spore and mycelial enzyme inactivation in air was first order with respect to the enzyme concentration. Increasing the O_2 tension to 1 atmosphere had no effect on

the kinetics of the mycelial enzyme inactivation indicating that O_2 did not inhibit the enzyme. The kinetic results suggest that the rate of enzyme inactivation may be dependent on the concentration of some complex which inactivates the reaction site. The purified Cu oxidase is similarly inactivated during catalysis and various theories have been proposed (29,43,44,64) to explain the mechanism of reaction inactivation. It is not possible though to extend these to the atypical enzymes since the nature of the prosthetic group is not known. The general theory of Lu Valle and Goddard (49) for enzymes that reduce O_2 to water to form a hydroperoxide ion suggests that the latter will tend to undergo spontaneous reactions leading to inactivation of the enzyme unless the free time before reacting with a donor molecule is very short. Some mechanism like this may explain the reaction inactivation of the mycelial and spore oxidases.

Available evidence would indicate that the cytochrome system is the main pathway of reaction with O_2 in plant tissues. Some tissues have been found, however, which are insensitive to cytochrome oxidase inhibitors, have an apparent low affinity for O_2 or in which cytochrome oxidase activity can not be demonstrated. Such findings would warrant a conclusion that a terminal oxidase system other than cytochrome is functioning. However, careful investigations have shown that CO, cyanide or azide insensitivity

can be due to inhibitor-resistant cytochromes (3,6,11,33, 55,84), that a low O_2 affinity can be due to gas diffusion limitations (23,79,84), and that an apparent lack of cytochrome oxidase activity can be a result of unfavorable extraction conditions (34,38,66,72). The presence of characteristic cytochrome abosrption bands has also been noted in most of the above cases. Although most results (34,72,83) suggest that the major terminal oxidase in plants generally is cytochrome oxidase, the question of whether ascorbic acid oxidase functions in the respiration or in some energy-yielding process in higher plant tissue is still unanswered.

In Darby and Goddard's (14) study of the respiration of M. verrucaria mycelium, they suggested that even though cytochrome oxidase was present, an alternative oxidase might be accounting for part of the respiration. This suggestion was based on the finding that the mycelial respiration was insensitive to high partial pressures of CO and to 0.0003M cyanide. The presence of a Cu-containing oxidase was considered unlikely since no polyphenol oxidase could be detected and various Cu enzyme inhibitors had little or no effect on respiration. The discovery of the atypical ascorbic acid oxidase which was insensitive to CO and was relatively insensitive to cyanide, azide and Cu chelators seemed to support Darby and Goddard's (15) suggestion of an alternate

oxidase. However, by selectively inhibiting the cytochrome system in the intact respiring mycelium with SN 5949 or antimycin a without inhibiting the ascorbic acid oxidase, it was possible to show that respiration in this tissue was dependent on cytochromes and not on ascorbic acid oxidase. Two further points of evidence supported this view. First, the surface-localized ascorbic acid oxidase could be completely inhibited without significantly changing the rate of endogenous respiration and, second, the O_2 affinity of mycelium tissue respiring in a moist gas phase was about 50 times higher than the O_2 affinity of the extracted ascorbic acid oxidase and was in the range reported for tissues utilizing cytochromes. Some additional evidence which favors the cytochrome system was Hilton's (36) evidence that the succinoxidase system in Myrothecium has the same essential role in aerobic respiration that has been established in many other organisms. Hilton also found that his mycelium preparations were more sensitive to cyanide than those of Darby and Goddard (15). In view of similar findings by other investigators (72) the lack of inhibition by CO and cyanide which Darby and Goddard (15) found would suggest either an excess of cytochrome oxidase in relation to cytochrome c or an alternate, cyanide-resistant pathway of electron transport through cytochromes of the b-type (b_3, b_7), or a cyanide insensitive flavoprotein. Darby and Goddard (15)

have observed cytochrome c absorption bands in heavy suspensions of mycelium while Goddard[1] has observed bands in the b, c, and a regions in isolated mitochondria frozen in liquid air. No data is available on the concentrations or turnover numbers of these cytochromes in the respiring mycelium.

All of this evidence, however, does not prove that ascorbic acid oxidase has no role in mycelial respiration since the experiments were done on mycelium only at one phase of growth. The enzyme could conceivably function only at certain periods of growth as proposed by James (39) and James and Boulter (41) for barley root ascorbic acid oxidase. Furthermore, measurements of the effects of inhibitors on O_2 uptake cannot prove to what extent the different oxidases participate in respiration in the absence of inhibitor. Also, no experimental evidence is available for enzymes in the mycelium, as there are in higher plants (5,54), which can link substrate oxidation to dehydroascorbate reduction. The presence of ascorbate could not be detected chromatographically in several attempts, but this needs further substantiation.

The action of an enzyme depends on the particular molecular structure of both the substrate and the enzyme.

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[1]Goddard, David R., Bloomington, Indiana. A.I.B.S. meetings. Personal communication. 1958.

Therefore, a study of substrate specificity aids in revealing which substrate atoms are concerned in the formation of the enzyme-substrate complex and what the general shape of the reactive site might be. Although nothing is known about the nature or geometry of the active site of the spore and mycelial enzymes, their specificity requirements allow some ideas about the number and points of substrate binding and the stereochemistry of attachment.

The high degree of specificity of the spore enzyme suggests a multipoint attachment involving the dienol group and the hydroxyl groups on carbon atoms 5 and 6 and possibly the C-1 carbonyl group. The dienol group is necessarily involved since electron transfer takes place here while attachment at the C-5 and C-6 hydroxyl groups on L-ascorbate is suggested since D-araboascorbate, 6-deoxy-L-ascorbate and hydroxytetronate were inactive. Further evidence supporting binding at these hydroxyl groups was that D-araboascorbate and 6-deoxy-L-ascorbate inhibited the enzyme. Proof of binding at the carbonyl group is lacking but a hydrogen bond between the enzyme and such an electronegative group seems highly probable. It would be of interest to test a C-1 substituted analog like imino-L-ascorbate to see if the carbonyl oxygen is required for activity. If there are 3 or more specific points of attachment of L-ascorbate to the enzyme there should be absolute stereochemical specificity

according to the theory of Bergmann and Fruton (4). This was shown to be the case for the spore enzyme by the fact that ascorbate analogs with the opposite C-4 configuration to L-ascorbate were inactive. Presumably then, a molecule such as D-xyloascorbate (III) would be inactive because the steric position of the side chain with respect to the ring has been altered and a 3-point attachment with all the groups in the right position in the enzyme molecule is impossible. If two optical centers are involved in a 3-point attachment, it might be expected that the enzyme would be stereospecific for both of them. This is apparently the case since inverting the hydroxyl group at the asymmetric C-5 position gave D-araboascorbate (IV), which was inactive. It was also consistent with the multipoint attachment theory that none of the 3, 4 or 5-carbon ascorbate analogs were active as substrates. The addition of a bulky primary hydroxyl group or a methyl group to the 6-carbon chain of L-ascorbate completely blocked activity as in the case of L-glucoascorbate, L-galactoascorbate and L-rhamnoascorbate. This is possibly a steric hindrance effect where the side chain is too long to fit into the binding region on the protein.

In the case of the mycelial enzyme, the side chain of L-ascorbate was not essential for attachment or activation since hydroxytetronate (XVI) was oxidized only 10 percent

slower than L-ascorbate. Although the side chain on L-ascorbate was not essential for activity, a change in the C-5 hydroxyl configuration or the substitution of a hydrogen atom for the C-6 hydroxyl group reduced the activity. This would indicate that the C-5 and C-6 hydroxyl groups function in binding and the formation of the activated complex and, if altered, affect the orientation of the substrate molecule at the reaction site, causing a decrease in rate. The lactone oxygen or ring also was not required since reductate (XVII), methyl reductate (XVIII) and reductone (XIX) were active substrates. Dihydroxyfumarate (XX) was presumably inactive due to the trans-dienol structure. Similar to the spore enzyme, the mycelial enzyme failed to oxidize any of the 6- or 7-carbon analogs with the opposite ring configuration to L-ascorbate. However, unlike the spore enzyme, the mycelial enzyme did not have an absolute specificity requirement with respect to the C-4 configuration since L-erythroascorbate (XV) was slowly oxidized. This would appear to be a case of steric hindrance by the primary alcohol group of L-erythroascorbate since the addition of one more carbon atom (D-xyloascorbate or L-araboascorbate) to the side chain led to complete inactivity while replacing the primary alcohol group with a hydrogen atom (hydroxytetronate) increased the relative activity from 23 to 88 percent. The oxidation of the 7-carbon analogs with the same ring

configuration as L-ascorbate showed that the chain length had no effect on activity as it did in the case of the spore enzyme. All of this evidence would suggest that the L-ascorbate molecule is attached to the enzyme on the side of the ring opposite the C-4 hydrogen atom. The possibility that the mycelial extract contained a significant amount of the spore enzyme was ruled out since several analogs were oxidized at the same rate as L-ascorbate. If any spore enzyme had been present, a lower rate of analog oxidation would be expected. Further evidence was that low concentrations (0.0007M) of D-araboascorbate had no effect on the rate of L-ascorbate oxidation by the mycelial enzyme while completely inhibiting the spore oxidase.

The specificity results were highly intriguing particularly since the enzyme changes its degree of specificity between two morphological forms of the same organism and yet retains almost identical properties otherwise. Whether this change is due to a structural modification of the protein or an actual difference in the amino acids making up the active site presents a complex problem. More information about this can only be obtained when the enzymes have been highly purified, if not crystallized.

SUMMARY

1. The mycelium of the fungus, Myrothecium verrucaria, contains an atypical ascorbic acid oxidase which has certain properties which are distinct from those of the atypical oxidase in spores of the same organism and of the Cu enzyme in higher plants. A method is described for the extraction and estimation of this enzyme.

2. At pH 4.5, the enzyme separated into a soluble and an insoluble fraction which had a somewhat different response to pH and several inhibitors.

3. Reaction rates were proportional to enzyme concentration up to approximately 350 microliters O_2 per hour. The enzyme was stable in solution at 30° C, had a pH optimum of 4.5 in phosphate-citrate buffer and a stoichiometry of one-half mole of O_2 used per mole of ascorbate. The K_m in air ranged from 0.00115M to 0.00135M ascorbate, increasing to about 0.0048M in O_2.

4. The concentration of O_2 in the liquid phase was a rate-limiting factor since the reaction velocity at 1 atmosphere of O_2 was only 90 percent of V_{max}. A calculated O_2 affinity (K_m) of 301 micromolar O_2 placed the atypical oxidase in the O_2 affinity range of the spore and Cu ascorbic acid oxidases.

5. The kinetics of the mycelial and spore ascorbic acid oxidases can be described in part by using the inte-

grated form of the Michaelis-Menton equation. An analysis of the plots of this equation showed that the integrated constant k'e was dependent on the concentration of ascorbate and O_2 and decreased with reaction time, apparently due to reaction inactivation.

6. Evidence from inhibitor and dialysis studies suggested that a heavy metal or a sulfhydryl prosthetic group is not involved in catalysis. The possibility that the enzyme has a metal or metal-flavin prosthetic group is still open.

7. Data from pH-activity curves and acid inactivation studies strongly indicated that the atypical enzyme is localized at the surface of the mycelium.

8. Two types of evidence demonstrate that the respiration of the intact organism is via the cytochrome system. The evidence is, first, that the affinity of the mycelium for O_2 falls well within the range of values for known cytochrome-dependent cells and tissues and, second, that the respiration of the mycelium can be completely inhibited with SN 5949 or antimycin a which specifically block the cytochrome chain but do not inhibit the atypical enzyme.

9. The ascorbic acid oxidase in the spores of M. verrucaria appears to be absolutely specific for L-ascorbate among 20 analogs tested while the mycelial and Cu enzymes are able to oxidize a wider range of structural types.

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ACKNOWLEDGMENTS

The writer expresses appreciation to Dr. F. G. Smith for his advice and aid during the course of this work. Appreciation is also extended to Dr. F. R. Duke and Dr. Dexter French of the Chemistry Department for their criticism of certain parts of the thesis. The encouragement and help provided by my parents, Mr. and Mrs. A. White, will always be remembered. These studies were supported by the Office of Naval Research, Department of the Navy, under contract (NR-103-198) with Iowa State College.

APPENDIX

Table 19. Inhibitor response of the mycelial enzyme.

Inhibitor	Conc. (M)	Expt.	Inhibition[a] (%)
Cyanide	0.01	18-11-56	30
		7-12-56	39
	0.001	18-11-56	20
		7-12-56	21
Azide	0.01	18-11-56	26
		7-12-56	35
	0.001	18-11-56	30
		7-12-56	35
CO-dark	19:1[b]	7-10-57	0
		27-12-57	0
CO-light	19:1	7-10-57	0
		27-12-57	0
8-Hydroxyquinoline	0.01	18-11-56	-7
		19-11-56	15
		5-12-56	34
Diethyldithiocarbamate	0.01	18-11-56	-26
		1-12-56	14
		5-12-56	1
		7-12-56	-4
	0.001	5-12-56	-10
		7-12-56	-26
	0.000001	1-4-57	0
Ethyl xanthate	0.005	5-12-56	34
		7-12-56	27
	0.001	19-11-56	4
Phenylthiourea	0.01	18-11-56	-20
		5-12-56	10
		8-12-56	16
Thiourea	0.01	5-12-56	11
		7-12-56	6

[a]Negative values indicate stimulation.

[b]95% CO: 5% O_2 at 1 atm. Control 95% N_2: 5% O_2.

Table 19. (Continued)

Inhibitor	Conc. (M)	Expt.	Inhibition (%)
Thioglycolate	0.01	7-12-56	18
BAL	0.001	7-12-56	5
o-Phenanthroline	0.001	2-12-57	0
2,2'-Bipyridyl	0.001	2-12-57	0
EDTA	0.001	4-2-57	19
		5-2-57	9
		14-2-57	20
	0.0001	29-1-57	20
		4-2-57	23
	0.000001	5-2-57	9
		14-2-57	12
o-Iodosobenzoate	0.01	5-2-57	-5
		14-2-57	1
Iodoacetamide	0.01	5-2-57	4
		14-2-57	9
Phenylarsenoxide	0.01	5-2-57	0
		14-2-57	2
p-Chloromercuribenzoate	0.005[c]	18-11-56	-53
		19-11-56	-16
		5-12-56	0
		7-12-56	-20
Arsenite	0.01	7-12-56	-3
	0.001	7-12-56	-8
Riboflavin	0.001	5-2-57	0
		14-2-57	0
		9-9-57	0
Isoriboflavin	0.001	16-2-57	0
		9-9-57	0
Atabrine	0.001	5-2-57	0
		14-2-57	0
		9-9-57	0
FMN	0.001	5-2-57	0
		9-9-57	0
		14-9-57	0

[c]Calculated molarity. Actual concentration was less.